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NUTRITION IN THE WAR

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DURING the first World War emphasis was on food. Now it is on nutrition, for this is the science concerned with the proper use of foods for the development and maintenance of healthy, well-nourished bodies. A great deal of factual information has been obtained in laboratories and clinics on the composition of foods and on the dietary requirements in health and in disease. Work along some of these lines has been intensified because it has been recognized that additional information was needed without delay for the handling of wartime problems. The principal activity, however, has been in the application of nutritional knowledge gained through fundamental research. This field of applied nutrition has required the active participation of workers in many other fields. It is the purpose of the present article to describe some of the current important aspects of applied nutrition and to mention also some of the problems that are being investigated at the present time.

SPECIAL PROBLEMS INTRODUCED BY THE WAR

Feeding the Army and the Navy.—"An adequate dietary," Colonel Paul E. Howe[1] has written, "is important for the soldier's health, morale, and effectiveness." In the Army, the immediate responsibility for procurement, distribution, and preparation of foods rests with the Quartermaster Corps, while the supervision of the diet of the soldier is the responsibility of the medical officers in conjunction with the officers of the line. The effort has been to have the American soldiers the best fed troops in the world, and, from reports of

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Colonel Howe, in charge of nutrition activities for the medical department, this goal has been attained for men in training or in service in the United States. The records show clearly that the individual soldier is receiving all of the dietary essentials in quantities exceeding the recommended daily allowances of the Food and Nutrition Board of the National Research Council.[2] It is gratifying to know that the tremendous problem of feeding the American troops is being handled so successfully.

Reports on the feeding of troops in overseas service and in combat areas probably will not be forthcoming until after the war, but there is every reason to believe that the men are well taken care of nutritionally. Rations for use overseas have been described by Wodicka.[3] Considerable interest has developed in the combat ration known as Field Ration K, developed originally in response to a request from the parachute troops, but of such merit that it has proved to be of general usefulness as an emergency ration. Three different units are provided in durable containers which together weigh about two pounds. The foods consist of different types of biscuit, canned meat with cheese, and a beverage concentrate. Chewing gum and cigarettes are also included. The meat, confectionery and beverage concentrates are different for each of the three meals. The nutritive value of these three meals has been designed to meet the requirements of the men. In the development of this ration, the Subsistence Research Laboratory of the Quartermaster Corps, located in Chicago and under the direction of Colonel R. A. Isker, has shown considerable skill and resourcefulness.

In the development of other special rations, a considerable amount of investigation is still under way, and, naturally, the results of such studies have not been published as yet. In this work, experts and officials of the Army, Navy, Marine Corps, Coast Guard, and Merchant Marine work in close cooperation with the Committee on Medical Nutrition of the National Research Council, the chairman of which is Dr. James S. McLester. This Committee is able to supply or obtain quickly the best available opinion on scientific questions and to plan tests for developing the answers to problems that need experimental investigation. Frequently these investigations are made in university laboratories under contract with the Office of Scientific Research and Development. The Navy recently has developed a Naval Medical Research Center located at Bethesda, Maryland, where nutritional and other questions related to the many special problems confronting the Navy can be studied. The scientific director of the Naval research laboratories is Dr. A. C. Ivy.

The emphasis on foods for combat troops is on keeping quality, compactness for ease in shipping, palatability, for no food is of any value unless the men will eat it, and on nutritional value, because it is recognized that if the foods do not provide the nutritional requirements of the men, recourse will have to be had to other methods of meeting these requirements. Industry has cooperated wholeheartedly and importantly with these newer developments, and, in the production of dehydrated foods especially, and in the perfection of proper packaging for special conditions, great advances have been made. Undoubtedly some of the newer developments will be useful to the civilian population after the war.

Much attention has been devoted to the requirements of foods for use by shipwrecked personnel, by troops in desert or jungle areas, or wherever an emer-

gency ration is needed in situations requiring conservation of the available supply of water. It is well known that the ingestion of protein foods and of inorganic salts subsequently involves the excretion of urea and salts in aqueous solution through the kidneys, thus depriving the body of some of its water. It would seem desirable therefore to reduce the protein and mineral content of these emergency rations to a minimum, or to avoid these foods entirely in favor of carbohydrate and fat. The use of candy containing citric acid, a stimulant for salivary secretion, has been developed. It would seem, however, that the formulation of suitable dietary schemes for use under such conditions is one which might well attract many investigators who are not now engaged in war problems. Is a man floating in a rubber lifeboat on a tropical sea better off eating nothing rather than eating fish which he may be able to catch? What should be the nature of the emergency foods provided? These are important nutritional questions.

There are many problems concerned with the feeding of men who are exposed to unusual environmental conditions, as in service on submarines or in airplanes. Bierman[4] has discussed the problems of aviation medicine concerned with air sickness, obesity, and the expansion of gases that form in the intestinal tract at higher altitudes. The condition of air sickness develops as a rule from acceleration during flight. It is considered to be a functional neurosis characterized by vomiting, nausea, apprehension, faintness, sweating, and sometimes prostration, and it may be precipitated or aggravated by the sight or odor of food. Men appear to be specially prone to develop air sickness on an empty stomach or after a heavy meal, and therefore Bierman has suggested that the meals should be light and served at four-hour intervals. Obesity in the aviator is undesirable, because fat affords an excellent storage for nitrogen and hence appears to aggravate the development of "bends" at high altitudes. The expansion of intestinal gases at high altitudes may cause intense discomfort and cramps that may persist for as long as twenty-four hours after returning to sea level. The elimination of gas-containing foods and beverages and those foods which are known to be particularly prone to develop flatus helps to avoid this difficulty, as does the feeding of frequent light meals. There is evidence that vitamin C is of considerable importance in enabling men to fly at high altitudes. The relationship of vitamin A to visual acuity, especially in dim light, is less clearly defined, but it is universally admitted that the rations of all military personnel should be thoroughly adequate with respect to all the vitamins and other dietary essentials, and a high content of vitamin A is desirable.

The value of the administration of additional vitamins when the diet itself is adequate with respect to these factors has been experimentally studied by Keys,[5] with negative results. On the other hand, there is accumulating evidence to show that the ability to perform work is decreased on low thiamine diets and improved following the administration of thiamine under these conditions. Thus on diets providing about 0.33 mg. of thiamine and 0.5 mg. of riboflavin daily, Foltz, Barborka, and Ivy[6] showed clearly that the work output was decreased and the subjects complained of lack of vigor, anorexia, and leg pain. When the deficient diet was supplemented with suitable quantities of yeast concentrate, providing thiamine, riboflavin, and other members of the B complex, there was a

prompt increase in the work output and a disappearance of the symptoms. At the Fatigue Laboratory at Harvard University, Egaña,[7] Johnson,[8] and their co-workers have also observed a close relationship between an adequate amount of thiamine and the ability to perform muscular work.

Problems Concerning the Feeding of Civilians in the United States.—The problem of meeting the civilian requirements for good nutrition is much more involved than the problem of feeding the armed forces. An excellent discussion of food supplies and public policy, food production and distribution is provided in the series of articles published under the title of "Nutrition and Food Supply: The War and After" in the Annals of the American Academy of Political and Social Science for January, 1943. Agencies of government have been established with the view of guiding agricultural production of foods and the distribution of foods in the wisest possible manner. The complexity of the problems involved may be illustrated by the situation which developed with corn in June, 1943. Because of prevailing ceiling prices, an editorial in the Southwestern Miller[9] states that farmers could anticipate obtaining about $1.50 for the pork produced from feeding about $1.00 worth of corn to hogs. As a consequence there developed a shortage of corn for human food purposes and for industrial needs and a tremendous increase in the hog population. The products of corn are needed not only for the manufacture of corn meal, cornstarch, certain breakfast cereal foods, and other foods such as corn sirup and dextrose, but also for the growth of significant amounts of the yeast used in the making of bread and for materials used in the manufacture of explosives, aluminum, magnesium, bronze, steel, and other war products. By the middle of June the War Food Administration had taken emergency steps to help relieve the situation and to enable essential war production to continue.

The rationing of foods has been developed to permit a more equitable distribution of foods in short supply. These include meat and butter and other fats, the supplies of which are being used in large part for military and lend-lease purposes, and sugar and coffee which must be imported. Canned foods have been rationed because they also are being used for military and lend-lease purposes and because any marked increase in production would involve the use of strategic materials and manpower. The reason for requiring ration coupons for evaporated milk is said to be in order to place this important food under control. Serving in an advisory capacity to help assure that rationing regulations of the Office of Price Administration will be in the interests of continued health of the people is the Civilian Food Requirements Branch of the Food Distribution Administration, of which Dr. Russell M. Wilder is Chief.

In the rationing of foods for civilians, consideration must be given to non-food uses, the requirements of food industries, restaurants, and institutions, and the requirements of consumers. The general policy has been to keep rationing regulations as simple as possible. There is a trend also to give preference in the materials allocated to industrial users to those manufactured foods where foods in short supply help further the use of those articles the supply of which is more abundant. In the allocation of relatively scarce items to industrial food processors, some consideration has been given also to the nutritional importance of the finished products.

People ordinarily learn about the nutritional value of foods from various sources, among which is advertising. Guidance of food advertising along educational lines long has been a concern of the Council on Foods and Nutrition of the American Medical Association. Realizing the importance of such work, the government has established a plan of cooperation with advertisers of food products known as the War Nutrition Program under the supervision of Drs. M. L. Wilson and W. H. Sebrell of the Nutrition Division of the Office of Defense, Health and Welfare Services of the Federal Security Agency, the administrator of which is the Honorable Paul V. McNutt. Early in 1943 the Nutrition Division was transferred to the U. S. Department of Agriculture and is now known as the Nutrition and Food Conservation Branch of the Food Distribution Administration. There has been a commendable trend on the part of food manufacturers and distributors to show in their advertising how the food article which they are selling fits into the scheme of an adequate diet for health. A new chart recently has been developed which describes seven basic food groups that should be included in the diet each day in the interests of good health. Thus advertisers of foods, in contributing to the National Nutrition Program, will be presenting the same basic information about the characteristics of an adquate diet.

Feeding Industrial Workers.—Recognition of the importance of the problem of feeding industrial workers is evidenced by the fact that at least three important committees have been established for the consideration of questions that arise in this field. The Food and Nutrition Board of the National Research Council has a subcommittee on the Nutrition of the Industrial Worker, and the Councils on Foods and Nutrition and on Industrial Health of the American Medical Association have formed a cooperative committee on Nutrition in Industry. The Office of Defense, Health, and Welfare Services maintains a division on Nutrition in Industry, which functions largely through the regional representatives and the State nutrition committees that have been established under the supervision of this agency. Close cooperation exists between this department and the Industrial Hygiene Division of the United States Public Health Service. A number of informative reports have been published and several research projects have been organized for the purpose of answering questions in this field. As Goodhart[10] has pointed out, a great burden of the work must be borne by local nutrition committees. The industrial nurse and the industrial physician often can help immeasurably in solving the immediate problems of an industry.

Surveys by Wiehl[11] of the diets selected by aircraft workers in California have shown the choice of foods to be exceedingly poor and it is reported that the condition has become worse rather than better. She found that if one assumed values equal to two-thirds of the recommended dietary allowances of the Food and Nutrition Board to be a minimum value for adequacy, the diets selected by these workers were deficient as follows: about 15 per cent were low in vitamin A, 25 per cent in calcium, over 40 per cent in riboflavin and in ascorbic acid. Medical appraisal of the nutritional status of such workers has afforded a comparable picture of deficiency, certainly a condition that is not conducive to continued maximum output of war materials. The principal weapon for overcoming such deficiency would appear to be education, but in the case of the

industrial worker, it is possible, and no doubt essential, to do more than that. It has been pointed out that in many plants, particularly the smaller ones, there are no facilities whatever for providing the workers with lunch or with mid-shift meals. Indeed the tendency is to keep the amount of time for eating to a minimum. The carrying of lunches or other packages into some of the larger war plants is prohibited because there are inadequate facilities for inspecting the contents of such packages. The manner in which some firms have met these problems and a discussion of other current nutritional activities in industrial establishments has been provided by Cowgill.[12]

Considerable interest has been aroused in connection with the question of giving all industrial workers vitamin preparations. In reviewing the evidence on this subject, a Joint Committee of the Council on Foods and Nutrition and of the Council on Industrial Health of the American Medical Association expressed the view that such indiscriminate administration was economically unwise, nutritionally unsound, and therapeutically irrational.[13] This Committee believes that much of the vitamins so administered would be wasted, and it expresses the opinion that vitamins could not correct all possible dietary deficiencies and holds to the conviction that the administration of vitamins is best handled under medical supervision, especially by the practitioner who has had an opportunity to examine the individual patient and thus to ascertain his requirements. More recently the argument has been advanced that the giving of vitamins now is desirable because of food rationing and because the vitamins in themselves will do no harm. Except for vitamin D, large doses of which may produce untoward effects, it is correct that the vitamins will do no harm. On the other hand, it is still questionable whether under the usual conditions contemplated for the administration of vitamins on a mass scale, they would accomplish as much good as would be accomplished by attention to the diet. Further, the supply of vitamin preparations is not inexhaustible, and increased production of thiamine, riboflavin, and ascorbic acid requires equipment, strategically important chemicals, and manpower.

THE NUTRITIONAL STATUS OF THE PEOPLE

A recent report[14] of the Committee on Diagnosis and Pathology of Nutritional Deficiencies, of the Food and Nutrition Board of the National Research Council, entitled "The Prevalence of Inadequate Diets and Deficiency States in the United States Together With a Consideration of Their Significance" asserts that every survey in the past decade has revealed widespread dietary deficiencies in the United States. It has been argued, however, that surveys of food consumption do not necessarily give a true picture of the condition of health of the people, and medical records have been cited to show that the incidence of malnutrition is almost insignificant. A discussion of the discrepancy of these two views has been presented by Jolliffe, McLester, and Sherman,[15] who have given reasons why national mortality tables and hospital admission rates tend to present an overly optimistic picture. Space does not permit a discussion of the results of newer methods of medical appraisement of the nutritional status, particularly because some of the methods are not yet firmly established; but probably every critic would agree that the diet on the whole is not as good as it ought

to be, and could be, and that the work of applying nutritional principles to practical human problems must be continuously carried on. The problem now as before is to teach people the principles of proper eating. There is insufficient evidence to judge the effects of rationing on the general nutritional status of the people in the United States. Some persons believe that the present situation has served to focus attention on the need of selecting foods on a nutritional basis. We are assured also that even with rationing the American dietary is adequate in quality and quantity of nutrients.

Great Britain.—A number of reports have indicated that the nutritional status of the people in Great Britain is, on the average, probably better than it was before the war.[16] If this is correct, it is likely that rationing schemes which guarantee the more equitable distribution of foods have been in large measure responsible. Since early in 1942 all infants, expectant mothers, and small children have been receiving daily some sources of vitamins C and D at least during the winter months. These groups also received extra rations of milk. Arrangements have developed so that people unable to afford these foods are given them just the same.

Although the overall picture is gratifying, it is true that some groups which before the war were better fed are in poorer nutritive condition today because of rationing. Body weight is said to be somewhat below the usual weight. The tuberculosis rate, however, has not changed markedly. There is some evidence that the incidence of anemia has increased, although the iron content of the diet probably is greater than it has been previously. The principal weakness in the British diet today, however, is in its vitamin C content. Although evidence of scurvy has not been observed, the people are living on diets very close to or below the minimum requirement of 30 mg., which is considered to be the amount necessary to prevent scurvy. The recommended daily allowance for the normal adult in the United States is considered to be 75 mg. Steps undertaken to correct this condition have been the encouragement of planting home gardens of green leafy vegetables and the utilization of unusual foods such as rose fruits which are relatively rich in ascorbic acid. In this connection, a recent report of Hunter and Tuba[17] in Canada is of interest. They found that edible products could be made from rose hips available in Canada, and it can be calculated that the expected harvest from Alberta would be sufficient to supply 140 million people (being equal to the population of the United States) with 100 mg. of ascorbic acid daily for a year.

THE OUTLOOK

With all of the attention that is being devoted to the subject, it is not overly optimistic to believe that the allied nations will come out of the wor with their nutritional status unimpaired and perhaps in many instances improved. Evidence of the fact that the nutritional point of view is taking hold is apparent to every close observer. Agricultural economists are beginning to speak occasionally of the nutritional yield of an acre of ground. Programs of agricultural production are laying considerable emphasis on the desirability of agriculture being geared to meet the nutritional requirements of the people. Laboratories have been established for the examination of soils and varieties of plants with

a view to ascertaining the kind of treatment of soils and the varieties of plants to select in order to reap the maximum nutritive value from the crops. Commercial processors of foods are becoming more and more concerned with the nutritive value of their products. Studies are being undertaken with a view to ascertaining the facts and then taking steps to conserve to the highest possible degree the nutritive value of the natural foods from which the processed foods are made. Notable in this connection is the program of the National Canners Association and the Can Manufacturers Institute on the nutritive value of canned foods.[18]

Fortification of foods has been placed on a sound basis,[19] as a result of considerations given by bodies such as the Council on Foods and Nutrition of the American Medical Association and the Food and Nutrition Board of the National Research Council. Of the common staple items there are only four classes of foods to which general approval has been given as vehicles for the administration of vitamins or minerals or both. These are enriched white flour and enriched white bread, which provide additional amounts of thiamine, niacin, and iron and, when supplies become available, of riboflavin, oleomargarine containing added vitamin A, iodized table salt, and vitamin D milk. The most recent of these developments has been the enrichment of flour and bread, and data now are available to show the value of this procedure.[20, 21]

In connection with the fortification of foods, mention should be made of the important work that the Food and Drug Administration of the Federal Security Agency, under the able leadership of Mr. Walter G. Campbell, has been doing. Definitions and standards have been developed for many common foods as well as for the fortified foods already mentioned, thus aiding "in protecting the public against exploitation by programs dictated more by commercial motives than by public interest."

Recently hearings have been concluded for the purpose of receiving evidence necessary to amend the standards for enriched flour and bread in accordance with recommendations of the Food and Nutrition Board of the National Research Council. Students of nutrition will find a study of the records of these hearings to be of great interest. The findings of fact relative to the higher enrichment levels for flour have been published.[21b]

A POSSIBLE WORLD-WIDE NUTRITION PROGRAM

Professor John D. Black[22] of the Department of Economics at Harvard University has written of the importance of nutrition in the postwar years in the following words: "All the knowledge of foods and nutrition that we now have must in due time be turned to the task of restoring order and building up a peace that will not be menaced by hunger and deficiency diseases." This concept of nutrition as a potent factor in harmony among nations in a postwar world was specially emphasized at the United Nations Conference on Food and Agriculture recently concluded at Hot Springs, Virginia. Probably the average person who has read only the newspaper accounts of this Conference is familiar with only two facts that were widely publicized: the fact that newspaper representatives were not admitted to executive sessions, and the report that considerable amounts of whisky were shipped to Hot Springs while the conference was

in progress. It does not seem to be generally known that there were representatives of 44 governments present, 9 of which were occupied by enemy nations. Though none of these delegates had the power to make agreements for the governments which they represented, it would seem likely that the agreements they did make as a result of the discussions at this meeting will be more binding than have been many of the treaties arranged by diplomats. The Conference probably was the first in history for the discussion of postwar planning while a war was in progress. Attention was directed, not to the requirements of individual countries, but to the rights of the individual human being. There were three sections to the Conference, and Section 1, which was concerned with problems of nutrition and public health, adopted a number of resolutions on May 29, 1943. Can anyone read the following account[23] of the first resolution adopted by this Section without realizing that nutrition is recognized as a factor of dominant importance for health?

The United Nations Conference on Food and Agriculture

having reviewed the information submitted by the several delegations on consumption deficiencies and the relation of food to health through the world and being deeply impressed by the dominant role played by adequate food in the reduction of sickness and death rates and the maintenance of health, the Conference

DECLARES:

1. That the first essential of a decent standard of living is the provision to all men of those primary necessities which are required to promote freedom from disease, and for the attainment of good health;
2. That the most fundamental of these necessities is adequate food which should be placed within the reach of all men in all lands within the shortest possible time;
3. That ample evidence has been presented revealing the existence of malnutrition in every country, with its inevitable consequences of preventable ill health; and

RECOMMENDS:

That the governments and authorities here represented:

(a) Immediately undertake the task of increasing the food resources and improving the diets of their people in accordance with the principles and objectives outlined in the findings of the Conference, and declare to their own peoples and to other governments and authorities here represented their intention of so doing;

(b) Undertake periodically to report to one another through the permanent organization recommended in Resolution II on the state of their national nutrition and on the steps being taken for its improvement.

It is of special interest to note the desire for immediate action in improving the diets of people and, in this connection, it should be stated that the delegates of the occupied countries were in favor of the resolution but desired that a footnote be added to explain that, obviously, countries in the hands of our enemies could not begin such a program right away.

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CLINICAL AND EXPERIMENTAL

THE PRINCIPLES OF PERCUTANEOUS ABSORPTION*

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ABSORPTIVITY of the skin has widespread implications in different branches of medicine such as physiology, pharmacology, toxicology, topical and systemic therapeutics, dermatology, industrial hygiene, balneology, and forensic medicine. Consideration of the fact that it may become important in war medicine also prompts a review of the subject at this time.

Electric Forces Regulating Absorption.[1]—On sending a direct current through the human body by means of metallic electrodes the initial intensity of the current drops considerably within fractions of a second, as if there were a sudden increase in ohmic resistance ("pseudoresistance of the skin"). This phenomenon is due to polarization, i.e., to the generation in the body of a current which flows in a direction opposite to that of the original current. The polarization current is produced in the epidermis exclusively. It fails to appear if the continuity of the epidermis is disrupted. It can be obtained in its full strength in the isolated epidermis.

If a direct current has to pass a membrane in a solution of electrolytes, a new electromotive force is produced, originating from the difference in diffusion-velocity of electrically positive cations and negative anions wandering through the membrane (electric diffusion potentials). Often the membrane is permeable only for either cations or anions, in which case a high potential difference may develop by the "electric double layer" on both sides of the membrane. This is the case with human epidermis which has a negative charge and is impermeable for anions.[2] Consequently aqueous solutions of electrolytes do not penetrate the epidermis.

If the isolated epidermis is used as a diaphragm in a vessel filled with water and a direct current is sent through the water, there is a movement of the water from the anode to the cathode.[3] This movement of water through membranes is called electroendosmosis. Its direction is towards the cathode if the membrane has a negative charge. By addition of salts or acids to the water the epidermis, or any other membrane with a negative charge, becomes discharged, and the movement of water is slowed down or stopped entirely. By addition of alkalies which increase the negative charge the movement is accelerated.[3]

The membrane effect of the epidermis is due not to the stratified structure of the Malpighian layer with its many layers, but to a single membrane being in

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This article is based on an earlier review of the subject[1] in which references previous to 1932 are quoted extensively. In this review the references will be restricted to publications of major importance of the period from 1920 to 1941.

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immediate contact with the electrolyte solution.[2] The horny layer cannot be regarded as a membrane in the electrophysiologic sense, because it represents a rather rough network of horny lamellae with large holes in it, and therefore is easily permeable for ions as well as for large molecules.[1] The electrical behavior of the skin is not influenced by extreme thinning or thickening of the horny layer. Therefore the seat of the polarization phenomena is to be placed in the transitional layers between cornified and noncornified epithelium (stratum granulosum and stratum lucidum). These layers are responsible for the polarization current ("pseudoresistance") and for the impermeability of the skin to electrolytes.[3] They are called the barrier of skin absorption. Being situated between the strongly acid stratum corneum and the slightly alkaline Malpighian layer, they represent an electric double layer with positive hydrogen ions on one side and negative hydroxyl ions on the other side.[1]

Functional changes alter the electrical behavior. Mechanical, chemical, thermic, and electric stimuli cause a diminution of the skin resistance ("local galvanic reactions").[4] This phenomenon is due to changes in the cell membrane causing increased permeability and decreased polarization currents. This condition can be demonstrated in any inflammatory lesion of the skin if the lesion is due to primary damage of the epidermis. Hyperemia due to vasomotor reflex does not change the resistance.

Misconceptions on Transepidermal Absorption.[1]—The surface of the skin is covered by a greasy layer consisting mainly of waxes and cholesterol esters.[5] The role of this covering as a factor in the impermeability of the skin for water and electrolytes has been overestimated. It is true that it hinders the wetting of the cornified surface, and so delays the penetration of water and aqueous solutions into the horny layer. Waxes and cholesterol esters are miscible with water, however, and therefore after prolonged contact with water, particularly with warm water, the permeation of aqueous solutions into the horny layer becomes possible. The keratin material swells in water as it is grossly seen on palms and soles where the thick horny layers show abnormal wrinkling and a whitish discoloration after a prolonged bath.

Similarly to the role of the greasy cover the role of the horny layer has been misinterpreted as a barrier for percutaneous absorption. Its large pores are permeable not only for water and aqueous solutions but for molecules of any size and even for gross molecule aggregates.

A third misconception is the idea that increased blood flow may facilitate the percutaneous absorption of substances which normally do not penetrate. This belief has arisen from the observation of qualitatively increased permeability in inflammatory lesions. Such increase is due to epidermal injury, however, and not to increased blood flow. If once the barrier of transepidermal absorption in the epidermis is broken through by any injury and a substance has entered the living epidermis, its absorption into the blood stream is no longer prevented. Increased blood flow may accelerate the rate of absorption but does not change the permeability of the skin qualitatively.

Absorption Through Appendages.[1]—The mechanism of percutaneous absorption as a whole becomes rather complicated by the presence in the skin of gland ducts and hair follicles which open on the surface in the form of visible

pores. The hair shaft does not adhere to the follicular wall. The space between is loosely filled with greasy, horny scales and contains air. This space is continuous with the duct of the sebaceous gland which empties the sebum into it. Any substance may penetrate this space and reach the duct of the sebaceous glands, and from there the gland cells. This pathway does not require passage through a stratified epithelium but leads through a canal filled with air to cell membranes, the permeability of which is higher than that of the transitory epidermal cells. Similarly, through the sweat gland ducts any substance may reach the secretory surface of the sweat gland cells after having passed the straight and the convoluted part of the duct. When a substance has reached the secretory cells, it easily enters the blood stream, even though it is true that a "basal membrane" has to be passed before the endothelium of the capillaries is reached.

Water and aqueous solutions do not enter the follicular and glandular pores by mere capillary attraction because of insufficient wetting and because the ducts are filled with air. Under pressure, however, the adherent air may separate and escape in form of bubbles as can be seen during a warm bath. In the case of inunction of ointments, compression of the air in the duct and absorption of the air through gland cells may take place.

Experimental Conditions and Methods.—As has often been emphasized, in experiments on percutaneous absorption the intake of substances through the respiratory tract and through the mucous membranes of mouth, rectum, and vagina has to be carefully avoided. Working with volatile substances, i.e., substances with relatively high vapor pressure, one must be particularly cautious to avoid errors in attributing absorption through the lungs to percutaneous absorption.

To prove that percutaneous absorption of substances foreign to the body has taken place, analytic-chemical methods should be preferred to any other method in demonstrating the presence of these substances in tissues, in the blood, or in the excretions. If, in lack of chemical methods, pharmacologic or toxicologic assays have to be used, such assays must be unequivocally specific for the substance in question. Histochemical methods, often used to prove the permeation of a substance into the skin proper, are sometimes of dubious reliability, and one should be particularly critical in evaluating the results obtained with such methods.

For experiments on the absorption of substances normally present in the body, the procedure of Hediger[6] is the method of choice. With some modifications it has been widely used by Bürgi[7] and his co-workers, with consequent valuable contributions to the subject matter. In this method the solutions to be examined are applied to the skin under glass bells with airtight occlusion, and changes in the concentration of the dissolved substances are estimated at certain time intervals in aliquot parts by quantitative chemical analysis of the fluid above the skin. Decrease of concentration indicates percutaneous absorption of the solute. The time curve of the rate of absorption can also be easily determined by this method.

Absorption of Water.—The bibliography of experiments on water intake through the skin of human beings is old and obsolete. Plethysmographic meas-

urements on a limb enclosed air tight in a water bath for several hours do not show any decrease in the volume of water.[1] It is conceivable that negligibly small amounts of water, within the error margin of the plethysmographic method, may enter the pores after having expelled the air. Practical experience, however, especially experience with the permanent "water bed" of the dermatologists, supports the assumption that the amount of water intake under normal pressure is extremely small, if any. Intake of water through the epidermis certainly does not take place. Whereas the horny layer swells throughout its whole thickness when a limb is kept in water, the living epidermis cells remain microscopically unchanged. It has been assumed that the pH of the cell proteins in the transitory layers between the cornified and noncornified epithelium is identical with the isoelectric point of these proteins which therefore are unable to swell in water.[1]

Absorption of Electrolytes.[1]—It can be stated as a general rule that electrolytes dissolved in water do not penetrate the skin of mammals. This impermeability is fairly well understood from the physicochemical point of view as discussed previously. The theory of pores in the cell membrane based on model experiments[8] furnishes a most satisfactory explanation for the impermeability of the skin for anions. Membranes with extremely fine pores adsorb selectively, depending on the electric charge, either cations or anions, and therefore these "ion sieves" are impermeable for electrolytes. Tiny canals in the cell may correspond with such pores.

The easiest way to demonstrate the impermeability of the skin to aqueous solutions of electrolytes is to apply to the skin salts, which normally are not present in the body, and to determine whether these compounds, if applied to the skin, are excreted with the urine. Salts with nonphysiologic cations, such as salts of lithium, cesium, rubidium, and strontium, were tested in this way. They were applied to the skin in aqueous solutions in the form of baths, sprays, etc., but none of them could be recovered in the urine.[1, 9] The impermeability of the skin for salts containing the physiologically occurring cations sodium and calcium has recently been proved by means of Hediger's method.[10]

Of the anions the iodide anion was tested most frequently. With iodides the experimental results were not unequivocally negative. In a few experiments traces of iodine were recovered in the urine after iodides had been applied to the skin in aqueous solutions. The iodide ion has a relatively good chance to enter the skin, because, in comparison with the chloride ion, it is an anion which increases the negative electric charge of the skin and its capacity of swelling in water.[1] Also, there is some evidence that small amounts of iodides may be oxidized to free iodine in the skin and be absorbed in this form.[11]

It has been shown histologically that if there is any absorption of iodides the route is through the appendages. Whether or not this route is passed, however, may depend on different conditions in the secretion of sebum, different degree of filling of the ducts, number and size of follicles, ducts, and glands, air content, etc. The mucous layer is absolutely impermeable for iodides. The same is true for salts in general. In principle their absorption through appendages cannot be said to be impossible. Practically, however, such an absorption does not amount to more than questionable traces.

Salts of bi- and trivalent cations, especially heavy metal ions with a low electromotive force, are partly lipoid-soluble and therefore absorbable by the skin. They will be dealt with among the other lipoid-soluble substances.

Percutaneous Absorption of Water and Salts in Different Species.[1]—In lower species intake of food and output of metabolic end products are partially functions of the skin but with the phylogenetic development of the animal world the physiologic importance of absorption through the skin has gradually decreased. There is only one basic difference between percutaneous absorption in lower and higher species, however, and this concerns the absorption of water. The skin of invertebrates, fishes, and amphibians does absorb water but that of mammals does not. As for electrolytes, the tegument of even the lowest animal is impermeable if the tegument is at physiologic rest. During the period of nutrition through the skin and during the excretion of metabolic end products there is a temporary increase of permeability.

Absorption of Lipoid-soluble Substances.—The "lipoid theory" of absorption (Overton[1]) has postulated that the cell membrane consists of lipoids because only lipoid-soluble substances may penetrate the cell. Also, according to this theory the penetration of a substance quantitatively depends on the degree of its solubility in lipoids. This theory was critically reviewed in an excellent article by M. H. Jacobs.[12] Also, he amplified the theory by relating the solubility in lipoids to the nonpolar structure of organic molecules.

As far as absorption in general is concerned, many exceptions to Overton's rule were found.[12] Practically no exception could be established for absorption through the skin, however. Qualitatively it has been proved that lipoid-soluble substances penetrate the skin, whereas substances insoluble in lipoids do not. Quantitatively, however, this rule cannot be carried through consistently. Some substances slightly soluble in lipoids penetrate with greater ease than others with higher lipoid solubility. Some authors suggest that a certain degree of solubility in water together with high solubility in lipoids is optimum for absorption through the skin.[13] In other words, a certain ratio of the solubility in water and in lipoids may be the decisive factor; e.g., alcohols, aldehydes (chloral hydrate, paraldehyde), and ketones (acetone), soluble in both lipoids and water, penetrate with great ease.

The idea that the permeability to lipoid-soluble substances is due to a sterol-phosphatide framework in the cell or in the cell membrane was reviewed and experimentally supported in 1936 by Starkenstein and his co-workers.[14] These authors confirmed and supplemented earlier incomplete observations[1] which indicated that any disturbance of the lipoid framework either by precipitation or by dissolution of cholesterol breaks up the barrier of skin absorption. Previous treatment of the skin with chloroform or ether, which dissolve cholesterol, or with saponins, which precipitate it, makes the skin permeable for water-soluble substances and diminishes the permeation for lipoid-soluble compounds. On the other hand, introduction of cholesterol into the skin, for instance with ointments, diminishes the permeability for water-soluble substances because the cholesterol wall has been strengthened.[15] In fact, from a table of my earlier work[1] in which the data on the absorption of potassium iodide from different ointment bases are listed, it is obvious that the absorption is poorer from bases containing cholesterol than from bases without it.

The number of lipoid-soluble substances which have been proved to penetrate through the intact skin into the general circulation is too large to be exhaustively listed in this article. Only a few of the medically important groups of lipoid-soluble substances will be discussed, namely phenolic compounds, lipoid-soluble heavy metal salts, hormones, vitamins, alkaloids and fatty ointment bases.

The percutaneous absorption of phenol was well known to physicians of the older generation. They witnessed severe and sometimes fatal poisoning following the application of carbolic acid to large areas of the skin. Such unfortunate events did not occur frequently, however, and the popularity of this kind of treatment was not decreased for some time. Many doubted its being dangerous at all. What is more, in a very recent discussion on the permeation of phenol the question again arose as to whether its external application may cause any systemic effect.[16] The reason for this uncertainty in a problem settled a long time ago may be found in the fact that phenol in high concentration coagulates the proteins of the skin, and combines with them to form large molecules, the lipoid solubility and penetration capacity of which are completely different from those of free phenol. It should be stated here as a general rule that *any substance when applied in a high concentration having a caustic effect is absorbed in relatively smaller amounts than when the same substance is applied in lower concentrations which do not elicit a gross response.* This has been proved not only for phenol[17] but also for a number of heavy metal salts[17, 18] and for hydrogen sulfide.[19] In addition, it has been shown that previous treatment of the skin with caustics[18] or with astringents[20] decreases the permeability to other absorbable substances.

Salicylic acid (o-hydroxybenzoic acid), the most frequently tested substance in experiments on percutaneous absorption, penetrates with extreme ease through the skin from aqueous and alcoholic solutions as well as from ointments. Lipoid solubility is a property of the nondissociated molecule and not that of the salicylate ion. Sodium salicylate which is ionized in aqueous solutions is not lipoid-soluble, and does not penetrate the skin unless there is enough carbon dioxide in the tissue to liberate salicylic acid from the salt.[1, 21] The esters of salicylic acid, used externally as antirheumatic preparations, are lipoid soluble and penetrate easily.[1, 22*]

Other lipoid-soluble phenol derivatives such as resorcin, hydroquinone, pyrogallol also enter the skin from any vehicle.[16]

Of the lipoid-soluble salts of heavy metals, mercuric bichloride was often found to be absorbed from its aqueous solutions through the unbroken skin. Similarly to phenol, however, its penetration is interfered with by its coagulating effect on proteins when applied in concentrations above 0.5 per cent.[18] From the literature of past decades one may obtain the impression that systemic mercury poisoning from external application of the bichloride occurred frequently when it was used in medicated baths. One has to consider that this method of therapy was usually applied in disorders involving broken skin, however, and that with the disrupted barrier massive absorption might have occurred.

*Wintergreen oil used as a solvent increases the absorption of other lipoid-soluble substances considerably.[17]

The penetration of the skin by lipoid-soluble salts of lead such as lead acetate and lead oleate has been shown to occur from aqueous solutions.[13] The amounts absorbed are small (0.1 to 0.2 milligram daily from a 10 centimeter-square area), and it has been doubted whether they have any importance in industrial hygiene in the matter of causing clinically manifest poisoning. These traces of lead, however, as an addition to the amount absorbed by the respiratory and digestive tracts, may do their share in the development of lead poisoning.

As does lead, copper enters through the skin into the general circulation when applied in the form of its lipoid-soluble fatty acid salts, for instance copper-oxyoleic acid.[23]

Salts of lead, tin, copper, arsenic, bismuth, antimony, and mercury tend to form compounds with the fatty acid radicals of the sebum and other fats of the skin surface. Thus an originally lipoid-insoluble compound in aqueous solution may be transformed on or in the horny layer into a lipoid-soluble substance. The percutaneous absorption, then, is due to, and quantitatively dependent on, this chemical transformation. The same reaction may take place outside of the body to a larger extent if the heavy metal salt is incorporated into ointment bases which contain fatty acids. This is one reason for the increased absorption of heavy metal salts from ointments as compared with their absorption from aqueous solutions.

Because of the great therapeutic importance in dermatology of ammoniated mercury (mercuric aminochloride) its percutaneous absorption has been the object of numerous investigations. Ammoniated mercury is insoluble in water, in lipoids, and in lipoid solvents. Its bactericidal effect is based on its adsorption to the cell walls and on an extremely slow dissociation of mercuric ions under the influence of the acid reaction of the horny layer and sweat.[24] Its absorption into the general circulation is practically nil.[1, 24-27] In careful studies Gibbs and his co-workers[28] have shown that any appreciable percutaneous absorption of mercury from ammoniated mercury ointments is due to gastrointestinal absorption following contamination of the oral cavity. In adults where contamination of the mouth is much less likely to occur than in children, systemic mercury poisoning is practically never seen after the use of ammoniated mercury ointments, even if the ointment is applied daily for months or years over the whole skin surface. A few isolated cases of such poisoning which have been described[29] apparently were based on extreme hypersensitivity to mercury with traces causing poisoning.

Percutaneous absorption of vitamins and hormones strictly follows the law that any lipoid-soluble substances are absorbed through the unbroken skin in measurable amounts. Most extensive studies on absorption of lipoid-soluble hormones have been carried out with sex hormones. These studies were reviewed by Eller and Wolff in 1940.[30] It seems to make scarcely any difference quantitatively whether these hormones are given in injections or in form of ointments because the total amount is absorbed in either case. The speed of absorption is different, however, the percutaneous absorption usually being slower. The lipoid-soluble vitamins A and D and the provitamin carotene behave in the same way as do lipoid-soluble hormones.[30] The fact that irradiation of the skin with ultraviolet rays is a substitute for the intake of vitamin D in the therapy of rickets is sufficient proof of the percutaneous absorption of vitamin D.[31]

In connection with the findings on the fast permeation of lipoid-soluble vitamins and hormones the old problem once more emerged as to whether percutaneous application of drugs has any advantage over the peroral and/or parenteral ways. Such an advantage is present only if one wants to create a slowly but steadily increasing level of the drug in the blood and tissues up to a certain maximum which may then be kept constant by daily inunctions. In the past the classical mercury inunction in the treatment of syphilis was advocated mainly on this basis, although similar absorption rates and concentrations have been achieved with intramuscular injections of insoluble salts (''depot effect''). Obviously such an advantage is not sought in the case of administration of hormones and vitamins. The aim of such therapy is saturation of the deficient organism. The saturation may be achieved through the administration of the lacking factor in any mode of application, and the speed of absorption is of no great importance. If anything, rather rapid absorption is desirable in vitamin and hormonal deficiencies.

Another reason for percutaneous drug application, however, is to create a higher concentration of the drug in a certain region of the body. It has been claimed that such an effect occurs, e.g., when salicyl-ester preparations are rubbed into the skin locally above the painful muscles in myalgia. There is little doubt that introduction of a drug into the skin may temporarily cause a relatively high concentration of the drug in the tissues close to the site of application, although in reference to muscles this never has been conclusively demonstrated. When in the treatment of deep-seated organs local percutaneous therapy appears to be superior to systemic administration, this superiority results from local vasomotor reflex processes rather than from higher drug concentrations.

In the skin itself a locally higher concentration at the site of application has been unequivocally proved by the unilateral swelling[32] and unilateral hyperpigmentation[33] of the nipples in response to unilateral application of estrogenic hormone. It goes without saying that the principle of topical dermatotherapy as a whole rests on the creation of a locally high concentration of drugs in the skin without appreciable systemic effect.

In a few unduplicated experiments the lipoid-insoluble and water-soluble vitamins, ascorbic acid,[34] and thiamin chloride (''betaxin''),[35] were found to penetrate the skin. These unexpected results should not be discussed here as no confirmation could be found in the literature.

From the extensive work on percutaneous absorption of insulin[36-40] one can conclude that under carefully controlled experimental conditions negative results are prevalent. Positive results are due either to skin injuries from shaving in animal experiments[38] or to a preparatory treatment of the skin with chemicals (alkalies,[40] benzine petroleum,[41] saponins,[42, 43] irritant plant extracts[44]) which pathologically increase the permeability of the skin.

The free alkaloid bases are lipoid-soluble and penetrate the skin. Their water-soluble salts do not penetrate. For instance, nicotine, strychnine, and opium alkaloids[17] are absorbed with great ease but not their sulfate or hydrochloric salts. The impermeability for the salts of alkaloids, like that of inorganic salts, is not absolute, but only traces go through, and these only after ap-

plication for many hours. The alkaloid base in the same amount and concentration may kill the animal in a few minutes. Of anesthetics nupercaine and pantocain were claimed to have the highest capacity of penetration.[45]

It has been known since 1879 that animal and vegetable fats and oils penetrate the skin via the pilosebaceous apparatus.[1] They enter the circulating blood after having been absorbed by the sebaceous gland cells. These cells excrete microscopically visible fat droplets as their function. In viewing their structure one is not too surprised to see the reverse process also take place, namely, the intake of roughly dispersed fat by their protoplasm.

It has also been known for a long time that mineral fats and oils enter the sebaceous glands and are absorbed systemically, although their absorption is not nearly so massive as that of animal and vegetable oils and fats. All these facts have been confirmed many times, most recently by Eller and Wolff[16] in detailed and careful experiments.

Penetration of animal, vegetable, and mineral fats through the epidermis proper never has been conclusively demonstrated. Fats are not "lipoid-soluble" in the narrower sense of the term, because they form not true but colloidal-disperse solutions with fat solvents.[1] Absorption from such solutions seems to be impossible for any living cell except sebaceous gland cells. Lanolin and paraffin are not absorbed by the epithelial cells of the intestines either,[1] and triglyceride fats are absorbed along this route only after they have been split into fatty acids and glycerin.

Absorption of Water-Soluble, Lipoid-Insoluble Nonelectrolytes.—Theoretically such absorption is not to be expected through a membrane which is impermeable to water. Experimentally it has been shown that sucrose[46] and galactose[47] do not penetrate from ointments, and it is highly probable that glucose does not either. It is regrettable that the available data on this problem are deficient and unsatisfactory.

Immunologic and allergic experiments seem to indicate that lipoid-insoluble antigenic substances which usually are large molecules or molecule aggregates may enter the skin from ointments.[48] This unexpected result, however, has been achieved only after rubbing the antigen-ointment vigorously into the skin for fifteen minutes. It has been stated that such treatment irritates the skin. A skin area treated in this way cannot be regarded as normal. It must also be considered that in the sensitized organism traces of the antigen are sufficient to elicit the immunologic reactions which have been used as indicators for the absorption of the antigens. That their absorption is not great in amount is indicated by recommendations to increase it either by previous treatment of the skin with benzene and petroleum benzine,[49] or by electrophoretic introduction,[50, 51] or by the use of the newly described penetrasols of Herrmann, Sulzberger, and Baer.[69]

Absorption of Gases.—Substances which are in the gaseous form at room temperature easily penetrate the skin with the remarkable exception of carbon monoxide which is insoluble in lipoids.[1] Ease of percutaneous absorption has been demonstrated for the following gases: oxygen,[1, 52] nitrogen,[53] helium,[53] carbon dioxide,[1, 10] ammonia vapor, hydrogen sulfide,[1, 19] hydrogen cyanide,

vapors of nitrobenzene, dinitrobenzene, dinitrotoluene,[1] and vapors of volatile aromatic oils.[17]

When hydrogen sulfide is applied to the skin in irritating concentrations, its absorption is relatively smaller than in low concentrations.[19] Experiments with aqueous solutions of hydrogen sulfide at various pH's[54] have revealed that the nondissociated H_2S molecule penetrates better than do the HS^- or NaS^- ions. Ammonium sulfide, however, seems to penetrate better than hydrogen sulfide.[55] If elemental sulfur is applied to the skin in ointment vehicles, its absorption occurs in the form of sulfides.[56] Cases of poisoning after treatment with sulfur ointments have been reported as due to this mechanism.[57] The manifestations are those of hydrogen sulfide poisoning.

The absorption rate of oxygen and carbon dioxide at constant temperature and pressure depends exclusively on the difference in the concentration of these gases within and outside of the skin.[1, 52] This absorption thus represents a simple physical diffusion process. This is probably true also for gases foreign to the body. The finer mechanism of this diffusion process has not been studied in detail. Its importance for toxicology and industrial hygiene is evident.

The Role of Vehicles.—Substances soluble in both water and lipoids such as salicylic acid or mercuric bichloride penetrate with great ease from aqueous solutions as well as from ointments or from fat solvents. Although usually the absorption from fats and fat solvents is more rapid and more complete than from water, this is not a general rule. It has been found, for instance, that estrogenic and androgenic hormones are better absorbed from aqueous vehicles than from oil.[30, 58] Such findings indicate that the permeation of a substance depends primarily upon its own (total or partial) lipoid solubility. The kind of vehicle into which it is incorporated and the solubility of the substance in the vehicle are of secondary importance.

The use of fat solvents, such as ether, chloroform, benzene, petrol-benzine, and, to a lesser degree, alcohol, usually enhances the percutaneous absorption considerably. This action is due not to the high solubility of lipoid-soluble substances in these solvents, however, but to the fact that these solvents in themselves increase the permeability of the skin. If the skin is treated with them in advance, i.e., previous to the absorption experiment, an increased absorption is subsequently observed from aqueous solutions as well as from any other vehicle. The classical experiments of Winternitz[1] with aqueous solutions of strychnine salts have proved this fact, and more recent observations have confirmed his results.[14]

Organic solvents may accelerate absorption by dissolving the greasy cover of the skin surface and of the follicular wall, thereby facilitating the wetting of these surfaces by aqueous solutions. This action is a minor factor, however. The main action of these solvents lies in their dissolving of lipoid compounds in the living cells themselves, thus disrupting the barrier of absorption.

Ointments.[1]—Ointments are fats or similar substances of a greasy consistency. They are applied to the skin with or without incorporated drugs, mainly for therapeutic purposes. Ointments can be spread over the skin in a continuous thin layer. This cannot be done with aqueous or alcoholic solutions, because their surface tension working against a low viscosity causes

diminution of the surface, and formation of drops with disruption of the layer results.*

In order to bring the thinly spread ointment layer close to the uneven surface of the skin, a pressure must be exerted. By the pressure the gas bubbles adherent to the pores escape sideways; deeper in the follicles and gland ducts, they become compressed and absorbed. Applying the same pressure, one will obtain a closer contact with the surface if the viscosity is low, i.e., if the ointment is flexible and supple. Ointments which have a high viscosity and cannot be spread in thin layers are called tenacious or tough.

Numerous data have been presented indicating that incorporation of a substance into an ointment increases its rate of absorption in comparison with the absorption from other vehicles. Such an effect is mainly due to the mechanical factors as described. One presses the ointment into the follicle, and thereby brings the substances more rapidly and in larger amounts to the absorbing surface of the sebaceous gland cells than is possible with other vehicles. The ointment bases are absorbed through the appendages exclusively, and therefore they can increase the absorption of incorporated substances only via this route. The importance of the mechanical factor is indicated by the fact that absorption is furthered more readily by supple ointments than by tenacious ones.

Although ointments are helpful in bringing the incorporated substance closer to the surface of absorbing cells, *there is no evidence that either ointments or any other kind of "vehicle" may serve as a transportation vehicle into the cell itself.*[14] If we choose a substance to which the skin is completely impermeable, e.g., sodium salicylate, we are unable to enforce absorption even with the most supple ointment and despite extreme pressure.[1] Data are available indicating that vehicle and incorporated substance are absorbed separately and at different rates of speed. It was found, for instance, that from the metallic mercury ointment (blue ointment) at first more of the ointment base and later more of the mercury was absorbed.[59] In addition, some experimental results indicate that the incorporated substance has to separate from the ointment base before it enters the cell; the greater the tendency to separation, the more intensive is the absorption.[60]

The problem of determining the composition of an ointment which would attain the maximum absorption of incorporated substances has received much attention. With the exception of the requirement of suppleness no general rule could be established. It has been shown that for various substances different ointment bases are optimal.[56] In general, however, the addition of cholesterol as an emulsifier does not favor absorption.[15]

Considering the importance of low surface tension and of thorough wetting of the follicular wall it was to be expected that application of the modern wetting agents (synthetic detergents) would enhance percutaneous absorption considerably. This was demonstrated recently by W. Duemling.[61]

Chemical reactions between substances and ointment vehicles may increase absorption, if by such reactions lipoid-insoluble substances are transformed into

*There are two surface tensions to be dealt with: one between skin and liquid, and the other between liquid and air. Both act in the same direction if the liquid does not wet the skin, as is the case with water. With alcohol the two tensions act in opposite directions; nevertheless, formation of drops occurs.[1]

soluble ones. The most common chemical reaction is the formation of fatty acid salts (soaps) from neutral salts.

Electrophoresis.[1]—By applying a galvanic current to the skin, substances can be introduced into the circulation which otherwise would not penetrate or would do so only in traces. "Electrophoresis," meaning electric transfer, is the most suitable term for this procedure because it does not involve any assumption concerning its mechanism. The term "cataphoresis" is properly reserved for the transport of colloidal particles in an electric field. The expression "ion transfer" or "iontophoresis" involves the assumption that only ions are transferred in one direction or the other depending on their charge. The term excludes the transfer of nondissociated molecules. "Electroendosomosis" is defined as the movement of a liquid through an electrically charged membrane under the influence of a galvanic current.

Attempts to utilize electrophoresis in clinical therapy can be traced back to the middle of the last century. Again and again it was rediscovered and was believed to accomplish more than other methods of application. In spite of some interesting results, such as histamine electrophoresis in myalgia and mecholyl iontophoresis in arthropathia, the therapeutic achievements of electrophoresis have been rather meager up to date. Possibly, more recent efforts to utilize this method in diagnostic procedures[50, 51] will be more successful.

The method of clinical electrophoresis is simple. The two poles of the galvanic current are connected with aluminum or zinc plates which are covered with gauze. The "neutral" electrode is soaked with some inactive electrolyte solution, usually tap water. It is firmly applied anywhere on normal skin, or it is clipped to the inside of an insulated vessel (enamel bowl) filled with tap water into which the patient's limb is immersed. The "active" electrode has a relatively small surface, thus producing a high electrical density, i.e., high milliamperage per square unit area. After its gauze has been soaked with the solution to be introduced, the active electrode is pressed and firmly fixed to the skin area to be treated, or anywhere on the skin surface if systemic effects are sought. A current of varying intensity from a few tenths of a milliampere up to 40 milliamperes (in the average 1 milliampere per 1 cm. square area) is then sent through the body for a period of time varying from a few minutes up to one-half hour.

When substances are introduced into the skin electrophoretically, both ion transfer and electroendosomosis are involved. The mechanism of ion-transfer is well understood. If the biologically active part of an electrolyte is a cation, the drug is applied to the positive pole (the anode), and the cation will travel toward the negative pole, i.e., towards the inside of the body. The inactive anion will travel in the opposite direction, i.e., from the soaked gauze to the metal electrode. Similarly, a substance with an active anion will penetrate to the inside of the body from the negative pole (cathode). Thus iodide and bromide ions are easily introduced from the negative pole, metal ions and alkaloid-cations from the positive pole.

Electroendosmosis, the transport of the liquid as a whole, however, interferes with this simple mechanism.[2, 3, 62] The skin has a negative electric charge, and therefore electrophoresis will effect a movement of water into the body from the positive pole, and toward the outer surface of the skin at the negative pole.

A gross clinical sign of this movement of water is a shrinking of the skin pores at the positive, and a swelling at the negative pole after intensive electrophoresis. In case of cation-transfer from the positive pole, electroendosmosis acts in the same direction, thus facilitating the absorption of the cation. The anion-transfer from the negative pole, however, is counteracted by endosomosis, which elicits a movement in the opposite direction.

Difficulties encountered in the introduction of dye anions ("acid dyes") were interpreted as being due to the lack of endosmosis in the same direction.[62]

The role of endosmosis was proved by experiments indicating that any factor which furthers electroendosmosis also favors electrophoretic introduction from the positive pole.[3, 62, 63] Such factors are the addition of sucrose or of alcohol to the aqueous solutions of drugs or dye stuffs containing an active cation, factors which are unfavorable for ion-transfer but enhance electroendosmosis. On the other hand, high salt concentration or acidification has an opposite effect; they are unfavorable for electroendosmosis and greatly hinder permeation through the skin.

In contrast to cation-introduction, the entrance of anions is furthered if electroendosmosis is suppressed by the addition of acids, of multivalent cations or by concentrated salt solutions. The addition of sucrose or alcohol inhibits the entrance of anions.

The effect of electrophoresis on percutaneous absorption has been studied mainly with respect to aqueous solutions of dyes and alkaloid salts.[1] A great many of the latter, such as salts of strychnine, morphine, and atropine which have no demonstrable effect when painted on the skin in aqueous solution, display their characteristic general toxic effects after a short time when they are introduced electrophoretically in the same amounts and concentrations. The electrophoretic introduction of other drugs may be demonstrated by local effects at the site of entrance. Local vasoconstriction by epinephrine salts, local sweating by salts of acetylcholine, mecholyl, and pilocarpine, and local anesthesia by salts of cocaine cannot be produced by simple application of their aqueous solutions to the skin but are easily achieved by introduction with the galvanic current. The decided effect of electrophoresis on skin absorption was demonstrated dramatically by Leduc in 1900.[1] A rabbit painted with a solution of strychnine nitrate survived this treatment without the slightest signs of poisoning, whereas another rabbit into which the same solution was introduced electrophoretically died in a few minutes from strychnine poisoning.

In spite of this remarkable effect of electrophoresis one has to keep in mind that application of galvanic current does not alter the essential principles governing skin absorptivity. Aqueous solutions of electrolytes enter the skin in traces through the appendages without the use of any current. If an aqueous solution of strychnine nitrate in high concentration is painted on the skin over large cutaneous surfaces and for a long period of time, it will pass the appendages in amounts which eventually will kill the animal.[1] The electric current serves only to increase this absorption through the appendages, but it does increase it tremendously. Under the conditions of medical electrophoresis, the current carries aqueous solutions through the appendages exclusively and not through the epidermis. With respect to dyes this principle was demonstrated as early as 1890,[1] and has often been confirmed since.[64, 65]

Under conditions favorable for electroendosmosis in the electrophoretic experiment, an aqueous solution may enter the Malpighian layer, but then, as shown in experiments with dyes, the solution penetrates through the follicular wall and not through the surface epithelium.[62] In other words, the barrier in the follicular epithelium which has a histologic and physicochemical structure similar to that of the surface epithelium, offers less resistance to the current than does the epidermal barrier. The latter forms the greater obstacle.

The electric current itself also travels through the appendages. If applied to the skin with electrodes which are soaked with saline solution previous to the absorption experiment, the current will increase the absorption of aqueous solutions which are subsequently painted on the skin. This effect is due to decrease of polarization. Decrease of polarization, however, is a minor factor if galvanic; i.e., noninterrupted direct current is applied.[1] The main effect of electrophoresis is the great increase of absorption through the appendages without qualitative change of the permeability of the skin.

Absorption Through Diseased Skin.[1]—The study of absorption through pathologically changed skin corroborates the basic rules of percutaneous absorption: (1) The thickness of the horny layer is of no importance (normal absorption occurs from ichthyotic skin); (2) the fatty cover of the skin is irrelevant (there is equal absorption from seborrheic and dry skin); (3) the barrier is in the epidermis (destructive lesions of the epidermis eliminate the physiologic barrier); (4) the most important way of absorption is through the appendages (decreased absorption occurs in scleroderma and other processes with associated atrophy of the appendages).

Recent Contributions.—Miescher[68] studied the penetration of water-soluble fluorescent dyes by applying them in different vehicles to the skin of laboratory animals. The depth of cutaneous penetration was noted by observing frozen sections under the fluorescence microscope. He found only diffusion into the upper loose horny layer, and thus corroborated the existence of a barrier at the lower level of the stratum corneum. Furthermore, he confirmed the observations indicating that the thickness of the horny layer is of no influence on the absorption through the skin.

The increasing use and extreme efficiency of topical application of sulfonamides in war surgery justify the interest in their percutaneous absorption. Recent experimental work[66] has shown that sulfanilamide penetrates the intact skin from organic solutions. Sodium and calcium salts of sulfathiazole enter the skin from aqueous solutions and from ointments; they also can be introduced electrophoretically.[67] On the basis of what has been said in this review, the lipoid-soluble sulfonamides may be expected to penetrate the skin from organic solvents and ointments. Aqueous solutions of the sodium salts should be applied to the intact skin only by electrophoresis. One cannot expect, however, that the electrophoresis of the sulfanilamide anion from the negative pole will be sufficiently effective, because of the antagonistic effect of the opposite direction of the forces of electroendosmosis.

Herrmann, Sulzberger, and Baer[69] have recently introduced new types of vehicles. These vehicles were designed to increase percutaneous absorption by combining the properties of fat or lipoid solubility, water solubility, surface-

wetting action, and actions as solvents and homogenizers. An example of such a combined vehicle and solvent consists of xylene (or derivatives), a synthetic wetting agent, antipyrine (for solution and homogenization), and propylene glycol. In their preliminary report, the authors have called vehicles of this type "penetrasols."

The authors state that these pentrasols accelerate the penetration of a great variety of substances through grossly intact human and animal skins (sulfonamides, heavy metals, so-called protein allergens, etc.). The reviewer is of the opinion that the effects reported exceed those to be expected by simple lowering of the surface tension and by thorough wetting of the surface (see. p. 19). The penetrasols developed by Sulzberger, Herrmann, and Baer may achieve their effects by attacking the lipoid frame of the living epidermal cells in a manner similar to the effect exercised by saponins.

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A SIMPLE LIVER PREPARATION ORALLY EFFECTIVE IN THE TREATMENT OF HUMAN AMYLOID DISEASE*

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ORALLY administered powdered whole liver can effect the resorption of experimentally produced amyloid in animals,[1] and can effect an apparent clinical cure in human beings with secondary generalized amyloidosis.[2] In the latter paper it was shown that the administration of such a liver preparation caused the amyloid to disappear, despite the continuing activity of the incitant inflammatory disease.

Many requests for the liver product or its method of preparation have come to us from physicians all over the country. The liver powder used in our original work is no longer available, the concern producing it having ceased operation. We have tried the leading commercial liver preparations, but have found them unsatisfactory in the treatment of secondary amyloidosis. We therefore believe that a brief description of a method of preparing a "liver cocktail," with which we have had results as good as those with the powdered liver originally used by us and which is inexpensive and easy to prepare, is in order.

METHOD OF PREPARATION

Liver obtained fresh from an abattoir is cleansed of its gross extraneous matter, especially gross blood vessels. It is then cut into cubes and passed

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through a Sep-Ro-Siv.* The very fine sieve of the latter is used, the liver forced through the machine several times in order to reduce the product to a fine, pasty consistency.

Fruit juices are added to wash as much of the liver as possible through the sieve of the machine. The finely mashed liver will protrude through the pores of the sieve, while any attached tough muscle, fibrous tissue, or large blood vessels will be extruded through the end of the funnel. This residue from the funnel should again be put through the machine several times, for it thereby helps clean the teeth of the grinder of considerable retained valuable liver mash. A spoon is finally used to free the sieve of adherent macerated liver.

The pressed tissue and the juice are collected in a bowl. Additional fruit juice is added to thin out the mixture, and this is then passed through a fine scoop sieve that can be purchased in any five-and-ten-cent or hardware store. The finished mash should be totally free from lumps of liver. It generally resembles a chocolate drink in color. The taste is that of the fruit juice used, is quite palatable, and is taken readily. A mixture of orange and pineapple juice is the vehicle most popular with our patients; any juice, however, may be used to suit the patient. The liver preparation should be kept in a cold refrigerator or icebox. In the event that the drink is too thick, additional fruit juice may be added to suit the patient's demand. At certain times of the year the liver may have a pungent taste. Washing one's mouth with unadulterated fruit juice, or chewing a few gum drops will easily and rapidly overcome this taste.

This procedure gives an approximate yield of 90 per cent. The dosage varies from the equivalent of ¼ to ¾ of a pound of liver daily. Wherever possible, the use of mash prepared daily is recommended. We have found, however, that a three-day supply kept at a temperature of 8° F. or lower is equally potent and palatable. After four or five days the juice becomes mildly sour. Two pounds of liver and one quart of fruit juice yield a total of about two quarts of the final mash, a supply sufficient for three days.

COMMENT

We have found this fresh whole liver preparation effective against amyloid. It has the advantage of being simple to prepare and of being far less expensive than any commercial product. It has the disadvantage of requiring a rather tedious and time-consuming procedure, and of daily preparation or, at most, at intervals of three days. Conscientiously adhered to, the yield in quantity and potency of the product is high, and the drink is pleasant in appearance and taste.

There should be no possibility of ingesting parasites in the liver if the material is obtained from a reliable slaughterhouse and the liver is carefully examined as it is cubed. We have found no parasites in any of our liver preparations after prolonged and careful search, including microscopic examination of the product.

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*Sep-Ro-Siv is manufactured by the Chisholm Ryder Co., Niagara Falls, N. Y., and is distributed through the American Utensil Co., 466 West Superior St., Chicago, Ill.

THE PROPHYLACTIC USE OF SULFANILAMIDE IN CHILDREN WITH RHEUMATIC HEART DISEASE*

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IN JANUARY, 1939, Coburn and Moore,[1] as well as Thomas and France,[2] published independently their observations on the prophylactic effect of sulfanilamide in rheumatic children. Stimulated by their favorable results, we began, in September, 1939, to administer sulfanilamide to a group of children attending our cardiac clinic. This communication is a report of the result of our study.

SELECTION OF CASES AND PROCEDURE

Observations were begun on children with inactive rheumatic heart disease who were attending the clinic. As the study progressed, a number of children were eliminated from the treated group and transferred to the control group because of (1) the appearance of mild toxic effects of sulfanilamide, or (2) lack of cooperation by either the child or the parent.

In all, 55 children were observed. There were 25 children in the treated group, and 30 in the control group. They varied in age from 6 to 14 years at the time of the beginning of the study. A study of Table I will show that, using the age of 12 years as the beginning of puberty, both groups consisted of almost equal numbers of pre- and postpuberal children.

TABLE I

AGE DISTRIBUTION AND SEX OF TREATED AND CONTROL PATIENTS

AGE	TREATED GROUP	CONTROL GROUP
6-8	1	3
9-11	12	9
12-14	12	18
Total	25	30

	TREATED GROUP		CONTROL GROUP	
	Boys	GIRLS	BOYS	GIRLS
Number:	12	13	17	13

From the outset, we followed one method of medication: 5 grains (0.3 Gm.) of sulfanilamide were given 3 times daily for a period of one week, and if no toxic signs or symptoms appeared, the dose was increased to 10 grains (0.6 Gm.) in the morning and in the evening. This dose was maintained for the remainder of the study.

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The drug was administered during two treatment seasons: (1) from September, 1939, through May, 1940, and (2) from September, 1940, through May, 1941. The actual period of treatment during each season was about eight months.

A complete blood count, sedimentation rate, and an electrocardiogram were obtained on each child prior to the initial administration of the drug. Throughout the period of observation, blood counts and sedimentation rates were obtained at two- or three-week intervals. At similar intervals, a careful history and examination were done to determine the presence either of ill effects of sulfanilamide or of evidence of rheumatic activity. The children in the control group were seen, on the average, every three months unless a rheumatic recrudescence occurred. In such instances, the children were seen more frequently.

TOXIC EFFECTS OF SULFANILAMIDE

We can corroborate the observations of Thomas and her co-workers[3] that the serious toxic signs and symptoms which are not infrequently noted following the administration of large doses of sulfanilamide are not seen when small daily doses are taken. Thus, cyanosis, severe hemolytic anemia or granulocytopenia, severe dermatitis, febrile reactions, were not seen in our series. Minor toxic effects, however, were observed in ten children. Recovery was prompt and complete in every child following cessation of therapy. Indeed, experience has shown that recovery may take place in some children even if the drug is continued. We did not deem it justifiable, however, to expose ambulatory children to the continued effect of sulfanilamide in the presence of even minor drug reactions.

It is of interest to record, briefly, the toxic effects observed in our group.

1. *Anorexia.*—V. A., a 14-year-old Italian boy, stated that he lost his appetite a few days after he began taking sulfanilamide. The drug was discontinued and was resumed three months later. Again his appetite diminished, and he lost weight. Sulfanilamide was stopped.

2. *Anemia.*—A moderate decrease in the hemoglobin content was found in four cases, accompanied in only one instance by a drop in the red blood cell count. In the latter case, the hemoglobin value fell from 12 Gm. to 8.6 Gm., and the red blood cell count from 4 million to 3,200,000. Upon stopping the drug these values rose to their premedication level. Three months later, the same child was again given sulfanilamide, but this time there was no change in either the hemoglobin or the red blood cell value.

3. *Leucopenia.*—In six children the total white blood count dropped below 5,000, but in only two of these children did granulocytopenia (mild in degree) accompany the leucopenia. Therapy was continued in the four children who showed a leucopenia alone, and in all, the leucocyte count rose to normal levels. The leucopenia observed in these children was mild: between 4,000 and 5,000 in three children, and 3,700 in the fourth child.

Therapy was discontinued in the two children who showed leucopenia and granulocytopenia. In both cases the blood counts returned promptly to normal.

4. *Rash.*—A mild maculopapular eruption was observed on the arms and legs of one child, and on the face of another. Both eruptions disappeared soon after stopping the drug.

5. *Allergic Reaction.*—Finally, one child, a 13-year-old Italian girl, complained of a severe frontal headache, which came on three hours after the initial dose of sulfanilamide and which persisted for five days until she discontinued taking the drug. One month later, she was again given sulfanilamide, but this time she did not complain of headaches. After a week of medication, however, "swelling of both knees, elbows, and shoulders" appeared during one night, only to disappear the next morning. She continued taking the drug. Two days later, her face became swollen and remained so for forty-eight hours. Sulfanilamide was discontinued.

RESULTS OF TREATMENT

Evidence of rheumatic reactivation appeared in both the treated and the control groups. In the treated group of twenty-five children, three manifested rheumatic recrudescences, and two of them terminated fatally. The details of these three cases are as follows:

CASE 1.—J. C., a 13-year-old Italian boy, has been a patient in our clinic for several years and suffered from rheumatic involvement of both mitral and aortic valves. After five weeks of sulfanilamide medication, evidence of congestive failure appeared. The drug was stopped and the boy admitted to the hospital. He died one month after admission in congestive heart failure. Autopsy findings corroborated the clinical diagnosis of chronic rheumatic heart disease with recent reactivation.

CASE 2.—C. C., a 13-year-old Italian girl, had been attending the clinic for several years and presented evidence of mitral insufficiency and stenosis, and of aortic insufficiency. She was given sulfanilamide from September, 1939, through May, 1940, without any untoward effects and showed no evidence of active rheumatic heart disease throughout this period. In November, 1940, she began taking sulfanilamide again and continued doing so until the first week of February, 1941. At this time she complained of headache, chills, and fever. A suspicious petechia was seen on the hard palate. The child was referred to the hospital with the diagnosis of subacute bacterial endocarditis superimposed on rheumatic heart disease. Subsequent laboratory and clinical data confirmed the diagnosis. She died about four months after admission to the ward. No autopsy was obtained.

CASE 3.—The third child (A. F.), a 12-year-old Porto Rican boy, showed evidence of rheumatic activity within one month following cessation of sulfanilamide therapy. He had taken the drug from October, 1940, until the beginning of May, 1941. Three weeks later, the sedimentation rate was found to be elevated and an apical diastolic murmur which had not been heard for more than a year, reappeared.

Among the thirty children in the control group we found three patients with rheumatic reactivation, one of whom died of congestive heart failure.

CASE 1.—S. M., a 14-year-old Italian girl, had mitral insufficiency and stenosis. In August, 1941, she had a sore throat, which was followed shortly by pains in her knees, ankles, and back. These subsided after a few weeks, but recurred in October, 1941. There was, however, no change in either the sedimentation rate or the electrocardiogram.

CASE 2.—K. M., a 14-year-old Porto Rican girl, had rheumatic insufficiency and stenosis. In October, 1940, her sedimentation rate became elevated, and she complained of pains in both knees.

CASE 3.—A. Z., a 13-year-old Italian boy, had mitral insufficiency and stenosis. In February, 1940, evidence of congestive heart failure appeared. This became progressively worse, and he was referred to the hospital. He died March, 1940. No autopsy was obtained.

COMMENT

It is readily seen from the foregoing résumé of our results that we could not demonstrate any prophylactic effect of sulfanilamide in our group. Two deaths and one recrudescence occurred in the treated group; one death and two recrudescences in the control group. Since it is probable that congestive heart failure and subacute bacterial endocarditis in children with rheumatic heart disease indicate the presence of an underlying carditis, our results show therefore that episodes of active rheumatic heart disease occurred with equal frequency in both the treated and control groups.

Nevertheless, we cannot conclude from our study alone, that sulfanilamide has no effect whatever in preventing recurrences of active rheumatic heart disease. Our series is not large enough to be statistically convincing. Furthermore, the general incidence of rheumatic infection during our study period of from September, 1939, through May, 1941, proved to be exceptionally low. It is therefore possible that given a larger number of cases, treated during a time when rheumatic recrudescences were frequent, that the effect of sulfanilamide may have been apparent.

Thomas and her co-workers[3] found that "in no patient under treatment did this serious malady (subacute bacterial endocarditis) develop," and raised the question "of whether sulfanilamide may prevent this complication as well." The course of events in our patient C. C., who developed this complication while regularly taking sulfanilamide, indicates that the use of this drug to prevent subacute bacterial endocarditis leaves much to be desired.

SUMMARY

1. Sulfanilamide in daily doses of 20 grains (1.2 Gm.) was administered to twenty-five ambulatory rheumatic children ranging in age from 6 to 14 years. As controls, we observed thirty children with rheumatic heart disease who were attending the cardiac clinic but who were not given sulfanilamide.

2. Observations were started in September, 1939, and were carried through to May, 1940. They were resumed in September, 1940, and stopped in May, 1941. Two periods each, therefore, of approximately eight months each comprised the time of the study.

3. Minor toxic effects were noted. These consisted of: (1) anorexia, one case. (2) Moderate decrease in hemoglobin content in four cases, accompanied by a drop in red blood count in one of the children. (3) Moderate leucopenia in four patients with concomitant mild granulocytopenia in two of them. The blood counts returned promptly to normal in every instance upon withdrawal of the drug, and in a few cases even while the drug was continued. (4) Mild maculopapular eruption in two patients.

4. Two deaths (one from congestive heart failure, and another from subacute bacterial endocarditis) and one rheumatic recrudescence occurred in the

treated group. One death (from congestive heart failure) and two recrudescences occurred in the control group.

5. In our series of cases sulfanilamide did not appear to be of value in preventing rheumatic recrudescences.

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CLINICAL CHEMISTRY

THE GLYCOLYTIC ENZYMES OF SYNOVIAL FLUID

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IN A previous publication[1] certain actions of glycolytic enzymes in cerebrospinal fluid obtained from patients with epidemic meningitis were discussed. Evidence was presented showing that while the larger part of the reducing sugar, glucose, in such spinal fluid disappears quite rapidly on incubation, the trace of fructose which is present[2] is destroyed very slowly. Other experiments showed that the enzyme which catalyzed the reaction was present in the cellular elements, and indirect evidence was presented which tended to prove that this enzyme was present in the polymorphonuclear leucocytes of the fluid. Further consideration of the question has shown that two objections can be made to these tentative conclusions. The first one is based on the nature of the material studied. The specimens in which the highest concentrations of leucocytes occurred were drawn shortly after acute exacerbations of the infection, and while tests designed to show the presence of meningococci gave negative results, still it is not impossible that organisms were present in small numbers and multiplied upon incubation of the specimens. Since meningococci react more readily with glucose than with fructose or, at least, form acid more readily from the first-named sugar,[4] the results cannot perhaps be attributed with certainty to the polymorphonuclear leucocytes. The second criticism is of an entirely different nature. Willstäter and Rhodewald,[5] in the course of investigations of the mode of formation of lactic acid from sugars, showed that, when buffered with phosphate solutions, glucose and fructose were about equally effective as sources for the formation of glycogen and lactic acid by suspensions of leucocytes.

Since these questions arose in connection with the earlier observations, it seemed desirable to carry out experiments in which (A) there should be available a convenient sterile source of large numbers of human polymorphonuclear leucocytes; (B) the sugars studied should be substances of known purity rather than those occurring in a complex biologic solution; and (C) the cells should be suspended in some biologic medium other than cerebrospinal fluid. In 1928 Cajori and Pemberton[6] described a patient with a sterile inflammatory reaction of the knee joint. On aspiration of this material it was found to be free from organisms but very rich in polymorphonuclear leucocytes. The authors carried out and reported the results of experiments upon the destruction of glucose added to this medium. Recently an opportunity presented itself of studying material from a similar case, and we decided to study the destruction of both glucose and fructose when added to this synovial fluid.

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The patient from whom the fluid was obtained was a Negro man 58 years of age. His chief complaint was that a swelling of the right knee was present and that he could move it only with difficulty. The knee was markedly swollen, not tender on palpation, and showed no sign of acute inflammation. The presence of fluid could be demonstrated on physical examination. Apart from this he had no complaints, and there were no other physical or laboratory findings which were abnormal. Serologic tests for gonorrhea and syphilis were negative. Material was repeatedly obtained from the joint space by aspiration. This synovial fluid was rich in protein and contained large amounts of mucin. It was apparently quite rich in fibrin, for in some instances a clot formed before studies could be initiated. Large numbers of leucocytes were present at all times, but the number varied quite markedly in different specimens. In at least one instance more than 1 c.c. of packed cells was obtained by centrifuging 10 c.c. of the fluid. Ninety per cent of the cellular elements were polymorphonuclear cells. The material was repeatedly examined for the presence of mircoorganisms. Tests were made, not only upon each of the six specimens used in the experiments to be discussed below, but also on at least six others. The methods of examination used included direct smears, cultures on many types of media, including those suitable for studying aerobic and anerobic organisms. Methods especially adapated for culturing the gonococcus and the tubercle bacillus were also employed. Specimens were also incubated for periods up to ninety-six hours and the incubated material later examined for organisms by direct smears and inoculation into various types of broth. Specimens of the fluid were also injected into guinea pigs. Positive results were not obtained by any of these methods in any instance. It seems certain that any demonstrable effects of cellular elements which might be found in studying this fluid were due to the leucocytes, for the number of organisms present must have been insignificant. The authors believe that the material was completely sterile.

In carrying out the experimental procedures, six different samples of this synovial fluid were used. The cell contents of these fluids varied markedly, but all were very high. In some studies the cells were first removed by centrifuging, and in others untreated fluid was used. These fluids were divided into portions of approximately 6 c.c. In many instances small amounts, usually 0.6 c.c., of concentrated sterile solutions of the purest glucose or fructose obtainable were added to these 6 c.c. portions and an equal amount of sterile water to an equal volume of the same material. These treated solutions were then mixed as thoroughly as possible to try to prepare equal concentrations of the cellular elements, and divided into three equal parts, each of which contained approximately 2 c.c. One of these was analyzed at once, one was placed in an incubator at 37° C., and the other placed in the icebox. An attempt was made to keep all solutions in each separate one of the six experiments identical, but this was found to be impossible because the leucocytes showed a marked tendency to clump together and because the gelatinous nature of the fluid, due to the presence of mucin, further prevented the preparation of suspensions of cells which were even approximately uniform. Even the three control preparations which made up integral parts of each separate test were not always exact duplicates of each other, although they seemed to show little variation in composition. The incubated and control solutions were precipitated by the zinc sulfate and sodium

hydroxide technique of Somogyi.[7] Reducing compounds were determined by the method of Folin and Wu[8, 9] and modified, when concentrations were low, according to the suggestions of Hubbard and Allison.[10] Fructose analyses were made by the resorcinol technique of Roe.[11] The solutions in which the determinations were finally carried out represented 1 to 10 dilutions of the original material. For the determination of concentration of sugar between 0.1 and 1.0 mg. per 100 c.c. (equivalent to concentrations of from 1 to 10 mg. per 100 c.c. of the original solutions), the method used for the determination of fructose was much more satisfactory than was the one used in determining reducing compounds. In each instance a culture was made of every solution upon which quantitative data were obtained, and all were found to be sterile.

TABLE I

REFERENCE AND EXPERIMENT NUMBER	MATERIAL USED	NOTES	SUGAR ADDED	INCUBATED (HR.)	INITIAL CONTROL (MG. PER 100 C.C.)	ICEBOX CONTROL (MG. PER 100 C.C.)	INCUBATED (MG. PER 100 C.C.)
A Average 6	As obtained		None	48	67	53	6
Average 5	As obtained		Glucose	48	162	156	10
Average 6	As obtained		Fructose	48	81	80	22
B IV	As obtained		None	24	71	73	7
IV	As obtained		Glucose	24	175	176	12
IV	As obtained		Fructose	24	81	87	22
IV	As obtained		None	48	71*	75*	7*
IV	As obtained		Glucose	48	155*	173*	12*
IV	As obtained		Fructose	48	81*	84*	10*
C VI	As obtained		None	48	51*	47*	20*
VI	As obtained		Glucose	48	138*	132*	2*
VI	As obtained		Glucose	48	220	197	116
VI	As obtained		Fructose	48	80*	79*	56*
VI	As obtained		Fructose	48	39	39	35
D IV	As obtained	Incubated for twenty-four hours before experiment	None	24	1	10	12
IV	As obtained		Glucose	24	105	70	29
IV	As obtained		Fructose	24	89	87	32
E I	Centrifuged		None	48	67	65	12
II	Centrifuged		None	48	81	89	89
III	Centrifuged		None	48	61	60	60
IV	Centrifuged		None	48	48	50	46
V	Centrifuged		None	48	69	64	50
Average 5	Centrifuged		None	48	65	66	51
Average 4	Centrifuged		Glucose	48	172	168	176
Average 5	Centrifuged		Fructose	48	84	84	87
F IV	Centrifuged	Washed cells added	None	96	45	44	20
IV	Centrifuged		Glucose	96	146	141	40
IV	Centrifuged		Fructose	96	73	80	31

The letters are given for reference to the text. Roman numerals show studies upon samples of fluid obtained by each of six separate punctures. Under "glucose" are given figures for total reducing compounds by the Folin and Wu method. Under "fructose" figures determined by the resorcinol reaction.

*Included in the averages listed under A.

The significant results obtained are given in Table I. In section A are given the average values for the studies in which sugars were added to the synovial fluid and the fluids incubated for forty-eight hours. Qualitatively the results of these experiments were all similar, and average values have therefore been presented. Quantitatively the results varied quite markedly. The most extreme variations encountered are given in sections B and C of the table. The average figures show that at least 90 per cent of the sugar originally present and 95 per cent of the glucose added were destroyed, while only between 70 and

75 per cent of the smaller amount of fructose added was lost on incubation. It seems probable that all the actual glucose present was destroyed in these experiments. The difference in the rate of destruction of these two sugars corresponds to the difference observed in the earlier experiments upon spinal fluid but is not as great as that found in those studies. The amount of the added fructose remaining in the solutions in the different experiments varied inversely as the concentrations of the cells present. The total reducing compounds reported as glucose, which were present after incubation, did not appear to be related to the cellular content. We believe that these figures largely represent nonglucose reducing compounds rather than glucose which had not been destroyed and that the irregularities noted are, in part, due to the difficulties inherent in determining such low concentrations of "sugar" and in part due to variations in the amount of some reducing compound extracted from the leucocytes in the different experiments.

Experiment B illustrates the difference in the destruction of these sugars when the solutions were incubated for periods of from twenty-four and forty-eight hours. The specimen studied in this instance was unusually rich in cells. Precautions were taken to try to insure the presence of equal suspensions of cells in all the solutions used, but it is probable that rather marked variations occurred. The added glucose was completely destroyed in twenty-four hours, while significant amounts of fructose, which was present initially in much lower concentration, were still found after forty-eight hours of incubation. The results obtained suggest that fructose was destroyed much more slowly during the second day of incubation than during the first one. This is true whether the figures are expressed in terms of the total amount of sugar or of the per cent of the total sugar which was present at the beginning of each twenty-four-hour period. Whether this apparent decrease in the rate of destruction was brought about by a decreased efficiency of the enzyme system, as some control experiments seemed to indicate, or resulted from a decrease in the amount of substrate, or from both cannot be determined.

Experiment C was designed to test the effect of variation in the amounts of the substrates upon the amounts of the sugars destroyed. In this instance varying amounts of the two sugars were added to a second specimen of fluid, which was much lower in cell content than was that used in Experiment B. In this experiment glucose in a concentration of 100 mg. per 100 c.c., or approximately a total of 2 mg. of sugar, was destroyed by the enzyme in 2 c.c. of fluid in forty-eight hours. Much smaller amounts of added fructose were destroyed. When 1.6 mg. of fructose (2 c.c. of an 80 mg. per 100 c.c. solution) were present, only 0.5 mg. was destroyed; when 0.8 mg. was present, only 0.08 mg. were destroyed. The difference between the reaction of the enzyme to the two sugars was striking, and in this experiment, as well as in the one just discussed (B) the amount of fructose removed appeared to decrease when the amount of sugar was decreased. In this instance also the difference in the rate of destruction was marked, whether it was expressed as amount of sugar or as per cent of the total amount of fructose initially present.

In Experiment D the addition of sugars to a sugar-free synovial fluid was studied. A specimen of fluid very rich in leucocytes (the same material used in experiment B) was incubated for twenty-four hours before the experiment was

begun. This experiment was the second one in which such a procedure was tried. In the earlier attempt all the glycolytic power toward both glucose and fructose disappeared when a speciman of fluid was incubated for one hundred hours. In this instance when sugars were added to the sugar-free fluid, 75 to 85 per cent of the 2 mg. of glucose and approximately 50 per cent of 1.8 mg. of fructose were destroyed in twenty-four hours. This experiment shows clearly that glucose was more rapidly destroyed than fructose when the sugars were added to incubated sugar-free synovial fluid. The destruction of fructose was approximately of the same magnitude as was that observed when fructose was added to the same untreated fluid (see results of Experiment B), but the destruction of glucose was apparently less marked than when the unincubated fluid was used. A number of factors affect the results which make us unwilling to draw definite conclusions from a comparison of these experiments. One of these is the difficulty in obtaining uniform cellular suspension. Another is the important part played in the calculations by the residual reducing compounds present at the time when the final analyses were made. It has already been pointed out that these varied markedly in different experiments, and in the present instance they appear to have been unusually high. Apart from such technical considerations, it is evident that two different effects may have been produced by the preliminary period of incubation. One is a destruction of the glucose originally present in the solution, so that in this instance the added fructose was the only sugar in the incubated material. The other is that the enzymes themselves may have been altered by the treatment, and this change in the enzyme may have had more effect upon the destruction of glucose than of fructose. Attempts were made to study this problem by modifying the conditions in various ways, but it was not found possible to determine definitely the cause of the difference between the results of Experiments B and D.

In Experiment E are given the results obtained by incubating synovial fluid from which cells had been removed by preliminary centrifugalization. In no experiment in which glucose or fructose was added to the cell-free fluid was there a significant change in concentration after incubation, and the average values only, therefore, have been included in Table I. The results of the experiments in which no sugar was added showed variations which were much greater than the probable accuracy of the methods employed, and the results of each have therefore been included. It is evident that not only were the average changes produced by incubation of the cell-free fluid smaller than those produced when cells were present, but also that in separate experiments the differences were marked. This seems to prove that the glycolytic enzymes affecting both glucose and fructose were present in the polymorphonuclear cells. However, two of a total of fourteen specimens (two of five specimens to which sugars were not added) showed a significant destruction of reducing compounds after forty-eight hours of incubation, and in one of these two instances, Experiment I, this destruction was marked. It seems most probable to us that in these two specimens enzymes had been extracted from the leucocytes, but the action of some other source of the glycolytic activity cannot be excluded. It should be noted that in the studies of Cajori and Pemberton[6] glycolytic activity of cell-free synovial fluid was demonstrated and that this activity was much less marked than was that of the uncentrifuged material.

In Experiment F are given results obtained when cells were removed from a speciman of fluid, washed in normal salt solution, and resuspended in the supernatant fluid. This experiment is interesting for a number of reasons. First, on the readdition of cells to a cell-free synovial fluid which possessed no glycolytic activity (as a control, see Experiment E IV, Table I), a distinctly demonstrable glycolytic activity was produced. Second, the destruction of glucose was more marked than was the destruction of fructose. Third, the process of centrifuging, washing and resuspending the cells had greatly reduced their enzymatic power, for the destruction of glucose and fructose was markedly less than the destruction in uncentrifuged material (see experiment C IV as control, Table I) although precautions were taken to have the concentration of the resuspended cells greater than that found in the original solution. In a second similar experiment carried out for forty-eight instead of ninety-six hours, no destruction of fructose and only a relatively slight destruction of glucose were observed.

Besides the experiments described above, cells from four of the six specimens, including those used in Experiment IV, were washed, resuspended in 0.85 per cent salt solution, and incubated for periods varying from forty-eight to ninety-six hours. No satisfactory evidence of a destruction of glucose or fructose added to such solutions was obtained in any of the experiments. This was probably due, in part, to the lessening of the enzymatic activity brought about by washing the cells illustrated by the experiments discussed in the preceding paragraph and in part by the unsuitability of the medium in which the cells were suspended. The suspensions to which no sugars were added did, however, give some fairly interesting results. When unincubated, sugar-free cellular suspensions were studied, no positive reducing or resorcinol reaction was obtained. After incubation the average "glucose" content of the four preparations was 12 mg. per 100 c.c., and the "fructose" concentration was 1.3 mg. per 100 c.c. The values in the specimens incubated ninety-six hours were higher than those studied after forty-eight hours. We believe that these apparent changes in the "sugar" content, which were almost certainly not due to glucose and fructose, were produced by disintegration of the cells. The same conclusion probably applies, we believe, to the small amounts of reducing compounds found in some of the experiments described in the earlier part of this paper.

It seemed possible that evidence might be obtained showing that glucose was changed into fructose or that fructose was converted into glucose; in other words, that a reaction simulating the Lobry de Bruyn effect[12] could be demonstrated during glycolysis by polymorphonuclear leucocytes. All specimens, both those containing glucose and fructose, as well as all control solutions, were therefore analyzed by both the reducing technique of Folin and Wu and the resorcinol technique of Roe. No evidence of the formation of one sugar from another was obtained. All variations of this type which were observed were of a magnitude which could be properly explained by the gradual increase in the control values found when suspensions of washed cells were incubated. Of course, the formation of small amounts of glucose from fructose under conditions which would lead almost immediately to their degradation would not be detected in such experiments.

CONCLUSIONS

When glucose or fructose was added to sterile synovial fluid which was rich in polymorphonuclear leucocytes, the sugars were destroyed. The rate of the destruction of glucose was at least twice as great as was that of fructose. The enzymes were present almost wholly in the cells, although they could occasionally be demonstrated in the cell-free fluid. The results confirm qualitatively those previously obtained upon cerebrospinal fluid.

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STUDIES OF PANCREATIC FUNCTION*

II. The Effect of Injury to the Pancreas or the Liver Upon the Amylase and Lipidase Content of the Blood

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QUESTIONS concerning the nature and origin of the lipidolytic enzymes of the blood are problems of major interest in physiology and clinical laboratory science. The earliest work of this kind was that of Hewlett[1] in 1904, who reported that after pancreatic injury there was in the urine an increase in the content of an enzyme which splits ethyl butyrate, or an "esterase"; also in one case there was observed in the urine an enzyme which splits the fats of olive oil, or a "lipase." Later, von Hess[2] (1912), also Hiruma[3] (1923), using

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ethyl butyrate as a substrate, obtained evidence indicating that there is an increase in the esterase content of blood after pancreatic duct ligation. In 1932 Cherry and Crandall[4] reported that after ligation of the pancreatic ducts in dogs, they could demonstrate in blood the presence of an olive oil-splitting enzyme (lipase), which normally did not appear in the blood stream. They also found that the activity of an enzyme normally present in blood, as measured by the hydrolysis of ethyl butyrate or tributyrin (esterase), did not show uniform increases after pancreatic duct ligation. They, therefore, concluded that the appearance in the blood of an enzyme which splits olive oil (lipase) is a specific indication of pancreatic injury.

Cherry and Crandall suggested that the term "lipase" be used to designate an enzyme which splits triglycerides of long chain fatty acids, as are in olive oil, and that the term "esterase" be used for an enzyme which splits simple esters, such as ethyl butyrate. They found, however, that tributyrin is apparently split by the same enzyme that hydrolyzes ethyl butyrate.

In 1934 Comfort and Osterberg,[5] using the Cherry and Crandall method for estimating serum lipase, reported elevated values for serum lipase in 95 per cent of cases of active pancreatitis, in 36.5 per cent of cases of carcinoma of the pancreas, and in 60 per cent of cases of carcinoma of the ampulla of Vater. In a later paper[6] these authors reported essentially the same results with this test, but in addition they found an increased serum lipase content in 97 per cent of cases of disease of the biliary tract in which there was an associated pancreatitis. These authors stress the requirement that the test must be made in less than ten days after the onset of pain. They conclude that the serum lipase determination is an efficient test for active pancreatitis, but less so for malignant disease of the pancreas.

In another paper[7] we have reported a procedure which includes greatly improved conditions for the determination of the lipidolytic enzymes of blood serum. With this method we have carried out an investigation in which conditions were set up to study the origin of these enzymes and their possible relation to pancreatic disease. Variations in the lipidolytic enzymes of the blood were followed with our method using five different substrates. Since Elman,[8] and others, have found the blood amylase a useful determination in acute pancreatic disease, it was decided to make parallel determinations of the serum amylase along with the lipidase estimations. For the amylase determination we used the method of Somogyi.[9] The cat was used as the experimental animal.

Four general types of experiments were set up. These were a study of the effect of the following upon the content of amylase and lipidolytic enzymes in the blood: (1) eserine and mecholyl stimulation; (2) ligation of the pancreatic ducts plus eserine and mecholyl stimulation; (3) pancreatectomy; (4) liver poisoning by chloroform. We were particularly interested in finding what conditions will elevate the blood concentration of these enzymes; whether, or not, these enzymes have an origin other than in the pancreas. Also, by means of the five substrates used, we hoped to obtain data showing which substrate could be considered the most reliable for studying pancreatic disease. A discussion of the experimental techniques and results is given.

ESERINE AND MECHOLYL STIMULATION

Experiments with eserine and mecholyl were carried out to standardize a method for stimulating pancreatic secretion which could be used for further experimentation. The procedure adopted was as follows: Cats were anesthetized by injecting intraperitoneally 30 mg. of nembutal per kilo of body weight. After anesthesia had developed, a control sample of about 18 c.c. of blood was collected by heart puncture. Eserine sulfate and mecholyl were then injected subcutaneously. A single dose of eserine sulfate, ranging from 0.125 to 0.50 mg., was administered, and the dosage of mecholyl was 0.05 mg. per kilo of body weight injected every half hour. After three to four hours of mecholyl administration a second sample of 18 c.c. of blood was collected from the heart.

Large samples of blood were collected to be able to carry out tests on five substrates for the lipidolytic enzymes, and also to carry out an amylase test. Since large blood samples had to be taken, it was obvious that only two samples of 18 c.c. each should be studied with an animal the size of the cat to obtain valid data; otherwise the blood analyses might reflect the result of hemorrhage. Therefore, in most of the data of this report only two blood samples were analyzed, the control and the experimental collected at the end of the experiment.

In Table I are the results of mecholyl stimulation in the presence of inhibition of acetyl choline esterase action by eserine. In practically every group of comparative analyses there is an increase in the amount of amylase and lipidolytic enzymes in the blood serum. A control on this experiment would be useful as some blood volume changes might take place as a result of the initial bleeding, the salivation, or other losses of fluids. The nearest to a control that could be obtained is the experiment with Cat 21, a depancreatized animal that had been healed with insulin. The increases observed in this cat are 12 per cent with tributyrin as substrate, 10 per cent with triolein, and 14 per cent with olive oil, values which are within the limits of error of this method. In view of these results with a depancreatized animal, it appears that the increases in blood enzymes observed after eserine and mecholyl stimulation of the intact animals are significant and that they reflect principally a pancreatic response. The increases in the lipidolytic enzymes noted are essentially parallel with the different substrates; hence no differentiation in these enzymes could be made. It is also of interest that the increases in amylase and lipidase are of the same magnitude.

PANCREATIC DUCT LIGATION WITH MECHOLYL STIMULATION

The results of experiments with pancreatic duct ligation are shown in Table II. In these experiments cats were anesthetized with nembutal and a control sample of blood was taken from the heart. A laparotomy was performed, and the pancreatic ducts were doubly ligated and cut between the ligations. Care was taken to ligate all ducts from the pancreas and not to obstruct the bile ducts. The abdomen was closed with sutures and skin clamps. Eserine and mecholyl were administered as described above. After two to five hours of mecholyl administration samples of blood were collected. All bloods were analyzed for amylase and for lipidolytic enzymes with five substrates.

TABLE I

THE EFFECT OF ESERINE AND MECHOLYL STIMULATION ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD OF THE CAT

CAT NO.	EXPERIMENT SUBCUTANEOUS INJECTION OF	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
9	Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 15 min.			140	180	76	93					39	66
	Same, repeated one day later			120	149	69	84	68	95			32	46
16*	Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 30 min.	912	1180	76	92	32	43	35	54			8	27
20	Eserine sulfate, 0.25 mg.; mecholyl, 0.05 mg. per kg. per 30 min.	1472	1938	93	112			51	88	35	47	27	56
22	Eserine sulfate, 0.25 mg.; mecholyl, 0.05 mg. per kg. per 30 min.	676	1336	77	100	43	56	51	49	12	27	9	20
21	(Depancreatized cat.) Eserine sulfate, 0.125 mg.; mecholyl, 0.05 mg. per kg. per 30 min.			123	141					45	50	42	49

*In experiment with Cat 16 experimental blood sample was removed two hours after start of injections.

For amylase values the Somogyi unit was used. The lipidolytic enzyme unit used here has been defined in another paper.[7] Values are expressed in units per 100 c.c. of serum. The experimental blood sample was collected approximately 3.5 hours after starting the injections.

TABLE II

THE EFFECT OF PANCREATIC DUCT LIGATION AND MECHOLYL STIMULATION ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD OF THE CAT

CAT NO.	EXPERIMENT	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
10	Pancreatic ducts ligated. Blood sample 24 hr. postoperative	420	1056	143	176	93	99	110	139			32	65
15	Pancreatic ducts ligated. Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 30 min. Blood sample 2 hr. postoperative	1057	1950	121	147	64	92	92	74			34	82
	Blood sample 5 hr. postoperative	1057	2050	121	443	64	411	92	374			34	109
19	Pancreatic ducts ligated. Eserine sulfate, 0.5 mg.; mecholyl, 0.05 mg. per kg. per 30 min. Blood sample 3.5 hr. postoperative			111	251					30	177		
23	Pancreatic ducts ligated. Eserine sulfate, 0.125 mg.; mecholyl, 0.05 mg. per kg. per 30 min. Blood sample 3.5 hr. postoperative	1195	1445	109	200	44	86	70	121	34	79	28	69

Values are expressed in units per 100 c.c. of serum. For amylase the Somogyi unit was used. The lipidolytic unit used has been defined in another paper.[7]

TABLE III

THE EFFECT OF PANCREATECTOMY ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD

CAT NO.	TIME AFTER PANCREATECTOMY BLOOD SAMPLE WAS COLLECTED	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
27	24 hours postoperative	976	911	108	102	57	44	73	67			25	31
29	4 days postop.	1726	644	99	384	53	96	76	280	31	101	26	106
	9 days postop.	1726	835	99	86	53	42	76	77	31	16	26	12
12	12 days postop.		1113		61		56		34				168
	19 days postop.		872		148		44		73				65
13	14 days postop.		180		59		40		47				30
	28 days postop.		1184		115		50		55				24
	42 days postop.		1075		135		59		91				35
	56 days postop.		1113		116		38		70				30
	70 days postop.		1035		141		81		97				59

Values are expressed in units per 100 c.c. of Serum. For amylase the Somogyi unit was used. The lipidolytic unit has been defined in another paper.[7]

This experiment was done to simulate conditions that probably exist in an active pancreatitis where obstruction occurs. A marked increase in amylase and lipidase was observed. The increases in amylase and lipidase are of essentially the same magnitude. These experiments show that pancreatic enzymes readily pass into the blood stream when the flow of external secretion of the pancrease into the duodenum is blocked. They do not indicate any difference in sensitivity of response of amylase as compared with lipidase. With the five substrates for lipidolytic enzymes, the order of sensitivity of response was the same as in normal cat serum. Tributyrin was split most rapidly; benzyl butyrate was next; and ethyl butyrate, olive oil, and triolein were the least sensitive to the splitting action of the enzymes present. Thus, a differentiation in the lipidolytic enzymes that appear in the blood serum after pancreatic duct ligation is not shown by these experiments.

THE EFFECT OF PANCREATECTOMY

The origin of the amylase and lipidase of the blood is a question of considerable importance. Somogyi[9] has presented convincing evidence that amylase is produced by the liver and it is well known that amylase is secreted by the salivary glands. The effect of pancreatectomy on the serum amylolytic potency has been investigated by a number of laboratories.[10-13] Reports upon this work are in good agreement in regard to a sharp postoperative drop in serum amylase concentration, but there are conflicting results as to whether or not the serum amylase value returns to normal with control of the diabetes by insulin. In as far as we know, the effect of pancreatectomy upon the concentration of lipidolytic enzymes in the blood has not been reported. We, therefore, decided that a study of the concentration of serum lipidase and amylase in cats following pancreatectomy would be of interest.

Pancreatectomy was performed under nembutal anesthesia, and the diabetes was controlled by insulin administration. The data obtained in these experiments are shown in Table III. The results in the experiment with Cat 27, in which blood was collected twenty-four hours after pancreatectomy, do not show variations that could be considered outside the limits of experimental error. Considering the other experiments as a group, the data show a decrease in the values for amylase and lipidolytic enzymes, following pancreatectomy, with a mixed return trend toward preoperative levels. No conclusion seems warranted from these results other than the very definite observation that amylase and lipidolytic enzymes do not disappear from the blood serum after pancreatectomy. This observation is in agreement with other work regarding the effect of pancreatectomy on the amylase content of serum and it appears to be a new finding concerning the lipidolytic enzymes of the blood.

The results of these studies, together with those of Table II, lead to the conclusion that the lipidolytic enzymes of the blood have an extrapancreatic as well as a pancreatic origin.

CHLOROFORM POISONING

It has been reported by several laboratories[4, 14, 15] that the liver does not produce an enzyme which splits the lipids of olive oil. We have obtained in-

TABLE IV

THE EFFECT OF CHLOROFORM POISONING ON THE CONTENT OF AMYLASE AND LIPIDOLYTIC ENZYMES OF THE BLOOD OF THE CAT

CAT NO.	HOURS AFTER INJECTION OF CHLOROFORM BLOOD SAMPLE WAS COLLECTED	SUBSTRATE											
		STARCH		TRIBUTYRIN		ETHYL BUTYRATE		BENZYL BUTYRATE		TRIOLEIN		OLIVE OIL	
		CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
17	18	1179	533	93	153	54	102	79	144			31	29
24	20	710	428	150	243	108	257	101	217	50	38	45	31
18	42	755	527	99	126	41	55	64	81			23	17

Dosage: 1 c.c. U.S.P. Chloroform per kg. of body weight. Values are expressed in units per 100 c.c. of serum. For amylase the Somogyi unit was used. The lipidolytic unit used has been defined in another paper.[7]

formation in agreement with this finding: a glycerol extract of liver was found to split ethyl butyrate, benzyl butyrate, and tributyrin, but not olive oil. In view of these findings, it was believed desirable to carry out liver poisoning experiments which might offer suggestions upon the influence of the liver, when diseased, upon the concentration of lipidolytic enzymes in the blood. The use of chloroform seemed indicated, since this compound has a primary toxic effect upon the liver.

In these experiments cats were anesthetized with nembutal, and control samples of blood were collected from the heart. Chloroform dissolved in olive oil was injected subcutaneously, the dosage being 1 c.c. of U.S.P. reagent per kilo of body weight. Samples of blood were collected at times varying from eighteen to forty-two hours after injection of chloroform. All bloods were analyzed for amylase and for lipidolytic enzymes by five substrates.

The results are shown in Table IV. The serum amylase decreased in all cases. The triolein and olive oil-splitting enzymes showed a very slight decrease, the significance of which is uncertain. A marked increase in the enzyme, or enzymes, of serum which split tributyrin, ethyl butyrate, and benzyl butyrate occurred. These results are of considerable interest because they show a differentiation in the lipidolytic enzymes of the blood; in an experimental procedure upon the intact animal, the enzyme, or enzymes, which split esters and tributyrin (esterase), were found to be increased in the blood serum, while the enzyme, or enzymes, which split triolein and the lipids of olive oil (lipase), remained at approximately the same or a slightly lower level. This is good evidence in favor of the assumption that there are in the blood two lipidolytic enzymes, or groups of enzymes; namely, esterases, which split simple esters and short chain fatty acid triglycerides, and lipases which hydrolyze triglycerides.

The most obvious conclusion concerning the increase in the esterase activity of the serum in chloroform poisoning is that it is a response to liver damage, although chloroform may have a toxic effect upon other organs or tissues which would be reflected by changes in the enzyme concentration of the blood. More work upon this phase of the problem should lead to interesting results.

DISCUSSION

The experiments described above were designed to get information upon the origin of the amylolytic and lipidolytic enzymes of the blood, and to study the relation of the pancreas and the liver to the circulating level of these enzymes in the blood. It was also thought that the use of five substrates for the estimation of the lipidolytic enzymes might yield evidence concerning the identity of these enzymes.

Stimulation with mecholyl in the presence of eserine, with and without pancreatic duct ligation, caused marked increases in the amylolytic and lipidolytic enzymes of the blood of cats. The increases in amylase and in lipidase were of the same magnitude, and there was no essential difference in the responses measured by the five different substrates for the lipidolytic enzymes. These results demonstrated that there is an increase in enzymes of pancreatic origin in the blood when a strong vagal stimulus is applied and when pancreatic duct occlusion occurs.

Pancreatectomy experiments resulted in some decrease in amylase and lipidolytic enzymes in the serum with irregular return trends toward normal levels. These experiments showed that both the "esterase" and "lipase" types of enzymes in the blood of the cat have an extrapancreatic source in addition to their pancreatic origin.

Experiments in which cats were poisoned with chloroform showed a decrease in serum amylase and a very slight decrease in the enzyme, or enzymes, of the serum which split triolein and the lipids of olive oil. Sera from the same animals, however, showed an increase in the enzymes which split tributyrin, ethyl butyrate, and benzyl butyrate. These experiments gave data which indicate a clear differentiation between the so-called "esterase" and "lipase" of the blood serum. Since chloroform has a primary toxic effect upon the liver, these results raise the question of whether or not the "lipase" determination, published by ourselves and others, might reflect the presence of liver disease as well as pancreatic injury. This question may be answered only by clinical studies.

SUMMARY

1. Experiments upon cats have been carried out which were designed to obtain information upon the origin and identity of the lipidolytic enzymes of blood serum.

2. Stimulation with mecholyl in the presence of eserine, with and without pancreatic duct ligation, caused marked increases in the concentrations of amylolytic and lipidolytic enzymes in the blood.

3. Pancreatectomy resulted in postoperative decreases in serum amylase and lipidase, with mixed return trends toward normal levels. These experiments demonstrated that the amylolytic and lipidolytic enzymes of the blood have an extrapancreatic source in addition to their pancreatic origin.

4. Chloroform poisoning caused a decrease in serum amylase. With respect to the lipidolytic enzymes of the serum, a dissociation of effects was observed: The triolein and olive oil-splitting enzymes showed a very slight decrease; and the enzyme, or enzymes, which split ethyl butyrate, benzyl butyrate, and tributyrin, were definitely increased. The latter behavior is evidence in favor of the existence of both "esterase" and "lipase" in blood serum.

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INTRAVENOUS INJECTIONS OF SOLUBLE TIN COMPOUNDS*

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THERE are no complete studies of the distribution of tin in the organs following intravenous injection of tin compounds. Accordingly, we adapted for toxicologic study the toluene 3,4-dithiol method developed by Clark[1] and extended to canned foods by DeGiacomi.[2] We also found it necessary to prepare tin compounds suitable for intravenous injection. The results of these studies are reported in this paper.

METHOD OF DETERMINING TIN

Preparation and Storage of Toluene-3-4-dithiol.—We obtained the compound, which is not on the market, by first preparing sizable batches of sodium toluene-3,4-disulfonate (3) and, then as the occasion arose, by converting small amounts of this to the dithiol (3). The distilled product was stored in a dark refrigerator.

Dithiol Solution.—A 0.2 per cent solution of dithiol in 1 per cent aqueous NaOH containing 0.5 per cent thioglycollic acid remains clear for several days when kept in the refrigerator. The solution should be discarded when turbidity appears.

Standard Sn Solutions.—Reagent grade tin metal is dissolved in concentrated HCl containing 0.5 per cent thioglycollic acid so that 1 c.c. represents 1 mg. Sn. This stock solution keeps indefinitely. For use in the analytic procedure, the stock solution is diluted with 0.5 per cent thioglycollic acid in dis-

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tilled water so that 1 c.c. represents 0.01 mg. Sn. This solution should be prepared fresh each day.

Range of Color.—In the range of 2 gamma to 20 gamma Sn there is a progressive increase in color, and the unaided eye readily detects differences of 3 gamma. In our experiments this sensitivity is 5 times greater than with the molybdenum-blue method.[4]

Procedure.—Fresh tissue (5 to 100 Gm.) is dissolved in a minimum of concentrated HNO_3, evaporated to dryness, and placed in a muffle oven at 500 degrees Centigrade until the charred organic matter is removed (usually twelve to sixteen hours). The ash is brought to fuming with 5 c.c. concentrated H_2SO_4, and heating is continued until solution takes place. After cooling, the mixture is neutralized with NH_4OH, acidified with HCl, diluted to 100 c.c. with distilled water, and finally brought to proper acidity by adding 2 c.c. of concentrated HCl. Hydrogen sulfide is passed through the mixture for 1½ hours, and the precipitate is collected on a Gooch crucible. The precipitating flask is rinsed with 30 c.c. of 10 per cent aqueous NaOH followed by distilled water. Each rinse is passed through the crucible. HCl is permitted to seep through until the filtrate is acid to litmus; then distilled water is passed through. The final volume is adjusted by evaporation or dilution to contain not less than 0.5 gamma nor more than 100 gamma Sn per c.c. To an aliquot of 5 to 10 c.c. in a tube there are added 1 drop of thioglycollic acid, 0.5 c.c. concentrated HCl, and 0.5 c.c. of dithiol reagent. A like amount of tin standard solution is treated in the same fashion. Both tubes are immersed in boiling water for ten seconds and the colors are compared directly by light reflected from a white background. The red color is discharged by various organic solvents (i-amyl acetate, i-amyl alcohol, n-butyl alcohol, di-n-butyl ether, carbon disulfide).

Factors Influencing Amount of Sn Recovered.—1. *Muffle Heating* for twelve to sixteen hours at 500 degrees Centigrade in the presence of H_2SO_4 or NaOH did not result in loss of Sn. The presence of HNO_3 resulted in lower values which were restored to practically complete recovery by heating the ash with concentrated H_2SO_4 to fuming, then neutralizing with alkali, and finally acidifying with HCl. The recoveries were made on duplicate samples in the range of 5 gamma to 100 gamma Sn.

2. *Sulfide Precipitation* resulted in a loss of Sn. With 50 gamma to 1 mg. the recovery was 96 to 100 per cent; at 10 gamma the recovery was 80 to 90 per cent; at 5 gamma it was 60 to 70 per cent.

3. *Presence of Animal Tissue* accounted for still greater loss of Sn in the lower ranges. With 100 gamma to 2 mg. the recovery was practically complete; with 50 gamma it was 90 to 94 per cent; with 5 to 30 gamma it was 60 to 80 per cent. Consequently the method has only qualitative significance if the sample contains 50 gamma or less and is doubtful at 5 gamma.

PREPARATION OF TIN COMPOUNDS

Stannic Citric Acid was prepared by adding 5 c.c. stannic chloride (anhydrous) to 30 Gm. of sodium citrate .11 H_2O dissolved in 40 c.c. of distilled water. The reaction product was precipitated by the addition of 10 volumes of 95 per cent ethyl alcohol. In one hour this was filtered and the white precipitate repeatedly washed with 95 per cent alcohol. Analysis by the dithiol method gave 20 per cent Sn. The product formed aqueous solutions readily

TABLE I

DISTRIBUTION OF TIN IN ORGANS OF RABBITS

NO.	MG. SN INJECTED		DEATH	KIDNEY				LIVER					LUNG					SPLEEN				
	TOTAL	PER KG.		MG. SN	% OF INJ. SN	CONC.	RQ	MG. SN	% OF INJ. SN	CONC.	RQ	% OF KID.	MG. SN	% OF INJ. SN	CONC.	RQ	% OF KID.	MG. SN	% OF INJ. SN	CONC.	RQ	% OF KID.
1	260	106	*	6.00	2.3	38.20	.36	5.11	2.0	4.30	.04	11.2	30.0	11.5	98.7	.93	258	.13	0.05	14.44	.136	37.8
2	250	104	*	8.33	3.3	45.51	.44	4.67	1.6	3.68	.03	8.1	4.1	1.6	26.4	.25	58	.20	0.08	15.38	.147	33.7
3	350	175	2d	83.3	23.0	474.0	2.7	4.41	1.2	4.91	.03	1.0	–	–	–	–	–	.26	0.07	16.25	.092	3.4
4	175	50	4d	7.5	4.2	37.50	.75	13.0	7.3	8.12	.17	21.6	0.80	0.4	2.66	.05	7	1.5	0.85	75.00	1.50	200.0
5	150	50	4d	7.5	5.0	25.00	.50	11.7	7.8	9.00	.18	36.0	1.50	1.0	3.95	.08	15.8	2.0	1.33	80.00	1.60	320.0
6	45.5	25	K 3W	0.43	0.9	3.43	.14	0.53	1.1	1.78	.07	51.8	0	0	0	0	0	.29	0.60	16.11	0.64	468.0
7	13.8	5	K 4W	0.05	.36	0.38	.08	0.31	2.2	0.26	.05	68.4	0	0	0	0	0	.003	0.02	0.31	0.06	81.0
8	15	5	K 4W	0.12	.80	0.81	.16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	Bladder urine 10.2; Bile 5.4; Small Intestine 7.59; Colon 5.47 Mg. Sn per 100 Gm. fresh organ																					
2			–	9.50				–		7.38		Mg. Sn per 100 Gm. fresh organ										
3			–	0				1.40		1.29		Mg. Sn per 100 Gm. fresh organ										
7			–	0				–		–		Mg. Sn per 100 Gm. fresh organ										
8			–	0				–		–		Mg. Sn per 100 Gm. fresh organ										

Abbreviations: Conc. = Mg. Sn per 100 Gm. of fresh organ.
RQ = Conc/Mg. Sn per Kg. of animal.
Kid. = Kidney.
* = End of injection.
d = days.
K = Sacrificed.
W = Weeks.

from which the sulfide was precipitated very slowly with H_2S. The aqueous solution (12.5 per cent) was acidic and 1 c.c. required 5.72 c.c. of N/10 NaOH for neutralization. For injection only neutralized solutions were used.

Stannous Citric Acid (20 per cent Sn), prepared with stannous chloride and precipitated as just described, flocculated when neutralized. Consequently the acid form was injected.

INTRAVENOUS INJECTION IN RABBITS

Stannic and stannous citric acids were injected instead of tin salts since the latter are liable to produce colloidoclastic shock. Neither compound in solution flocculated with rabbit serum, 10 per cent egg white solution, nor with Ringer's solution at pH 6.0, 7.0, or 8.5. Neither did they hemolyze mammalian erythrocytes.

Death usually occurred during intravenous injection of solutions of either compound in about twenty minutes; when approximately 500 mg. (100 mg. Sn) per kg. had been introduced at the rate of 0.5 c.c. per minute. Tremors appeared when 25 per cent of the fatal dose had been administered; flaccid paralysis when 50 to 60 per cent had been administered; and terminal tonic and clonic convulsions toward the end of the injection. The viscera, especially the lungs, were severely congested. The acutely fatal dose is practically the same as for sodium citrate. Animals which received only 50 per cent of the acutely fatal dose responded with a brief period of excitement followed by lethargy, from which they recovered and showed no further symptoms until death four days later. At autopsy the kidneys were pale and granular. No edema was seen. Animals which received 25 per cent or less of the acutely fatal dose survived until sacrificed about one month later.

ORGAN CONCENTRATION AND FATE OF THE INJECTED TIN

Our data in Table I are too few to warrant detailed deductions but point to the similarity of tin to heavy metals,[4, 5] especially bismuth. In general the amounts of Sn in the kidneys, and lungs, the intestines, and the bile diminished progressively with the time after injection; but those in the livers, and the spleens increased before they decreased. Animals that died during injection, or shortly after, had Sn in all organs and fluids that were examined; but after three weeks only spleen, liver, and kidney had amounts sufficient for analysis. The kidney probably loses the metal last. The kidneys, except in Rabbit 1, had a considerably greater concentration of metal than did other organs. The concentrations in the intestines and the bile did not exceed those of the urine. The spiral valves of the intestines were not involved in detoxification since they did not have high tin concentrations and had no deposit of tin sulfide.

CONCLUSIONS

1. Dithiol is applicable for quantitative analysis of tin in tissues.

2. The method is quantitative for samples containing at least 50 gamma of tin.

3. The sojourn and concentration of tin resemble heavy metals, especially bismuth.

4. The organs tolerate relatively high concentrations of tin.

5. Tin is excreted rapidly following intravenous injection.
6. Stannic citric acid and stannous citric acid possess low toxicity.

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LABORATORY METHODS

GENERAL

DEMONSTRATION OF TUBERCLE BACILLI IN TISSUE BY FLUORESCENCE MICROSCOPY*

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AT THE present time there is much interest in the identification of *Mycobacterium tuberculosis* in smears of pus, sputum, and body fluids by staining with a fluorescent basic dye, auramine O, and examination of preparations in ultraviolet illumination. The staining procedure is similar to the Ziehl-Neelsen method with the substitution of the fluorescent dye, auramine, for basic fuchsin. Thin smears are stained with carbolauramine usually at room temperature. The preparations are decolorized with acid alcohol, washed with water, and examined dry without a cover glass by means of the fluorescence microscope in ultraviolet light. Hagemann[1] in 1938 introduced this method. Since this publication, a number of papers[2, 3, 4, 5, 6, 7, 8, 9, 10] have been written confirming his results. These authors consider this method superior to the Ziehl-Neelsen technique for the detection of the tubercle bacillus in smears of sputum, pus, and body fluids for the following reasons:

1. The staining method with auramine is simpler than with basic fuchsin (Ziehl-Neelsen). Smears can be stained with carbolauramine at room temperature, while steaming carbolfuchsin is usually necessary for the Ziehl-Neelsen method.

2. Auramine, a di-phenyl methane basic dye, is probably a more specific stain than basic fuchsin for acid-fast organisms. (The number of tubercle bacilli stainable with carbolauramine appears to be greater than with carbolfuchsin.)

3. More tubercle bacilli are detected in smears stained with carbolauramine than with carbolfuchsin. Self-luminous, yellow fluorescent tubercle bacilli stained with the fluorescent dye, auramine, are more easily seen against a dark background than red-stained bacilli on a blue background. Bogen[8] reports in 1,000 duplicate smears stained in parallel by the Ziehl-Neelsen and fluorescent methods, there were over 20 per cent more positive tests by the newer technique.

*From the Medical Department and Scientific Bureau of Bausch & Lomb Optical Co.
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Lind and Shaughnessy[9] in an examination of 1,123 smears by means of the Ziehl-Neelsen and fluorescence methods found 106 to be positive. Twelve of these preparations, positive by the fluorescence method, were negative by the Ziehl-Neelsen method. They also state that because of the larger areas covered and ease of examination, the time required was reduced to one-fourth or one-third that required for the Ziehl-Neelsen method.

4. Smears are examined for tubercle bacilli in much less time. An oil immersion objective with a 10X eyepiece provides the usual magnification (930X) for examination of smears stained by the Ziehl-Neelsen method. Tubercle bacilli stained with auramine appear as yellow, fluorescent bodies on a dark field. The fact that the bacilli become secondary light sources serves to increase the contrast. Therefore lower magnification may be used, and thus a larger field may be examined at one time.

The fluorescence staining method as used for smears of sputum, pus, and body fluids may be modified for the detection of *Mycobacterium tuberculosis* in fixed tissue. The staining technique, equipment, and method of examination are as follows:

STAINING METHOD

1. Fix tissue in 10 per cent neutral formalin for twelve to twenty-four hours. Commercial formalin may be neutralized by the addition of marble chips a few days prior to fixation.

2. Dehydrate completely, imbed in paraffin and cut sections on a microtome at 6 to 10 microns. After removal of paraffin with xylol and passage through alcohol to water, place sections in carbolauramine. Since paraffin will fluoresce, it is essential that it be entirely removed by xylol.

Auramine O (National Aniline)	0.1 Gm.
Distilled water	100 c.c.
Liquefied phenol U.S.P.	4 c.c.

The auramine O is dissolved in the distilled water by shaking strongly in a flask. Gentle heating will hasten its solution. When the auramine is dissolved, the liquid phenol is added.

The application of heat shortens the staining time and usually increases the intensity of the stain. The following staining times are suggested:

Room temperature	30 minutes
Incubator 37° C.	15 minutes
Paraffin oven 55° C.	10 minutes
Gentle steaming of slides flooded with carbolauramine	3 to 5 minutes

4. Wash the preparations briefly in several changes of distilled water.

5. Decolorize in 95 per cent ethyl alcohol containing 0.5 to 1 per cent hydrochloric acid until the preparations are quite colorless. The time for decolorization does not usually require more than forty-five seconds. Two to three changes of acid alcohol should be used for this decolorization.

6. Wash preparations in distilled water.

7. Transfer to 0.1 per cent aqueous methylene blue, certification No. 18 (National Aniline), C. I. No. 922. Sections are stained from thirty seconds to one minute. As with the Ziehl-Neelsen method, the methylene blue removes any carbolauramine not removed by the acid alcohol with little sacrifice of the intensity of the auramine in the tubercle bacilli. In addition, by staining the tissue the methylene blue masks the natural fluorescence of the tissue and forms a dark background for the yellow, luminous tubercle bacilli.

8. Rinse in distilled water.

9. Dehydrate, clear in xylol, and mount in xylol-clarite. Most mounting media, including balsam, fluoresce in ultraviolet light. Tissue preparations are usually examined dry without a cover glass or mounted in nonfluorescing media such as distilled water, glycerin, or medicinal mineral oil. Although xylol-clarite fluoresces slightly, preparations mounted in this medium are permanent. In addition, its refractive index 1.515 is more favorable for the examination of stained elements.

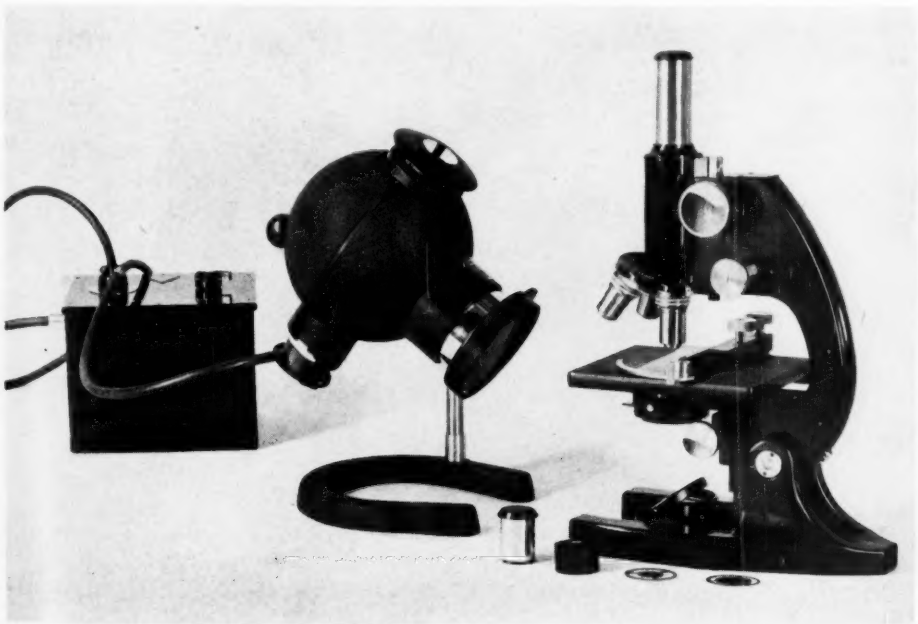

Fig. 1.—Equipment for fluorescence microscopy. Mercury vapor lamp, ultraviolet transmitting filter, Abbe Condenser 1.25 N.A., dark field stop 15 mm. diameter.

Any microscope (see Fig. 1) having a magnification of 430X and suitable for the examination of biologic material can be used for this work. The standard medical microscope is suggested. Although not absolutely necessary, the ordinary silvered glass mirror should be replaced with an aluminized mirror, for both glass and silver absorb ultraviolet.

As a source of illumination a mercury vapor lamp with iris diaphragm and filter holder is suggested. An ultraviolet transmitting glass filter and a dark field stop complete the equipment necessary for examination.

METHOD OF EXAMINATION

The preparations are best examined by enclosing three sides of the microscope with a shield to exclude extraneous light. A drop of nonfluorescent sandalwood oil or Shillaber's Immersion Oil is placed between the slide and condenser. The beam of light from the mercury vapor lamp is centered on the microscope mirror, and the condenser is focused up and down until a uniform distribution of light is obtained in the microscope field. The best condition is obtained when the substage condenser forms an image of the front aperture of the lamp in the plane of the specimen. A dark field stop is placed in the slotted ring below the substage condenser. Low power magnification with 5X or 10X eyepiece and 16 mm. objective is usually employed for the initial exploration. The observer should search for any luminous yellow bodies on a

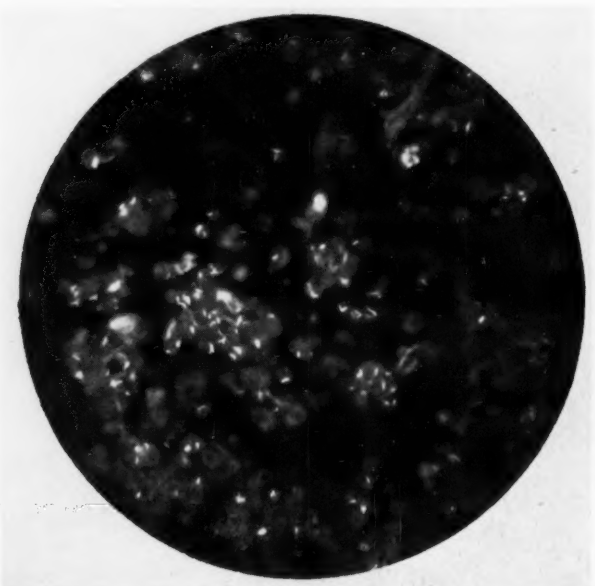

Fig. 2.—Photomicrograph of tubercle bacilli in lung tissue. Apochromatic objective 8.3 mm., 0.65 N.A. Eyepiece 10× Hyperplane. Magnification 400×. Exposure time: 7½ minutes.

dark field. Provided the staining technique has been properly employed, the only luminous structures in the field will be tubercle bacilli. A higher magnification, 5X or 10X eyepiece with 4 mm. objective, should now be used to inspect the morphology more closely. Tubercle bacilli appear as thin, yellow, luminous, slightly curved rods on a dark background. (See Figs. 2 and 3.)

Variations in the method of examination will be suggested to the observer. For example, one may prefer to use an 8 mm. objective with a 20X ocular, or for higher magnifications, a 15X or 20X eyepiece with the 16 mm. or 4 mm. objective. Although an oil immersion objective is not necessary, it may be used even in an undarkened room with a 5X or 10X ocular using Shillaber's immersion oil, sandalwood oil, or synthetic oil of wintergreen between the slide and objective. If desired, preparations may be first focused and examined in visible

light by using a daylight filter or a ground glass and blue filter. This will show the nuclei of the tissue cells and other basophilic elements that may be present, stained blue by the methylene blue. In the case of smears of sputum, pus, and body fluids, the blue staining of the nuclei of leucocytes can be observed. If many tubercle bacilli in the preparation are concentrated at one spot, it is even possible at this time by partially closing the diaphragm and using a high dry 4 mm. objective to locate this auramine-stained area in visible light. It appears as a pale yellow area on the methylene blue background. According to Gage,[11] a long card smeared with anthracene in a strong solution of cane sugar and dried makes a good detector for focusing the beam upon the aluminized mirror, and a similar preparation on a corex glass slip serves well for making sure that the ultraviolet is passing up through the condenser to the place where the preparation is to be located. A piece of fluorescent glass or cellophane might be used just as well for the same purpose.

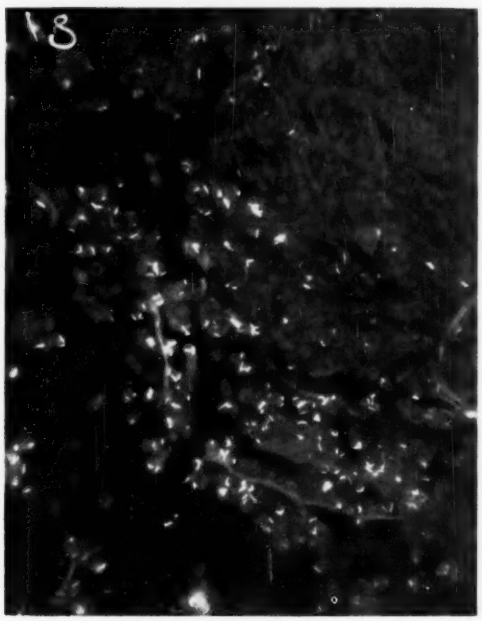

Fig. 3.—Photomicrograph of tubercle bacilli in lung tissue. Achromatic objective 4 mm., 0.65 N.A. Eyepiece 7½ Hyperplane. Magnification 400×. Exposure time: 30 minutes.

Distilled water or glycerin does not fluoresce, and although their refractive index is not that of immersion oil, they can be used in place of sandalwood oil or Shillaber's Immersion oil to make contact between the slide and condenser.

SUMMARY

A staining method and equipment* for the detection of tubercle bacilli in tissue is described. Tissue sections 6 to 10 microns are stained with carbol-auramine solution and decolorized with acid alcohol. Preparations are counterstained with methylene blue, dehydrated, cleared, and mounted in xylol-clarite

*The equipment, including mercury vapor lamp, dark field stop, filter, metal shield, and aluminized mirror may be obtained from Bausch & Lomb Optical Co., Rochester, N. Y.

for permanent preparations. Distilled water, glycerin, medicinal mineral oil, or Shillaber's Immersion oil may also be used as mounting medium, provided the cover glass is sealed with shellac or paraffin wax. The preparations are examined with ultraviolet radiation using low power magnification (50X or 100X) and a dark field stop for the initial examination. Higher magnification (430X) is necessary to check the morphology of stained elements. Tubercle bacilli appear as thin, yellow, luminous, slightly curved rods on a dark background.

The staining method and fluorescence microscope have also proved satisfactory for the detection of *Mycobacterium tuberculosis* in sputum, pus, and body fluids. One of us, Erich Loewenstein, has successfully used this method for the detection of lepra bacilli in smears.

We acknowledge with thanks the assistance of Mr. J. V. Butterfield of the Scientific Bureau of Bausch & Lomb for making the photomicrographs shown in this paper.

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AN IMPROVEMENT OF THE SEROLOGIC KAHN REACTION IN THE SPINAL FLUID*

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THE Kahn test is carried out with ease and gives very reliable results in serum. In spinal fluid, however, various difficulties arise, so that other methods have been preferred. These difficulties result mainly from the attempt to concentrate the effective antibodies through precipitation with ammonium sulfate, as recommended by Kahn himself. In this method, the spinal fluid is mixed with an equal quantity of a saturated solution of ammonium sulfate and well shaken, thus precipitating the globulins. Inactivation is obtained by heating the mixture at 56° C. for twenty minutes. It is then centrifugated at high speed and the supernatant fluid is poured off.

The following factors may lead to inaccuracies:

1. A certain quantity of antibodies may be dissolved in the supernatant sulfate solution, and when the latter is poured off, they are lost for the reaction.

2. The spinal fluid may be poor in globulins, so that, although it may be rich in antibodies, only a fraction of them is precipitated together with the present amount of globulins.

3. The salt concentration should always be the same and should be reproduced exactly. The considerable quantity of ammonium sulfate which may remain in the precipitate obviously changes the salt concentration in which the reaction is going to take place.

4. Finally, the scarcity of albumin in the spinal fluid may lead to a pseudopositive reaction, for the flocks of the antigen which are normally dissolved in serum may remain suspended in a fluid poor in albumin.

Considering that the inaccuracies mainly arise from the attempt to concentrate the antibodies in the spinal fluid, we developed a method for spinal fluid, similar to the one used for serum.

Reagents employed:

I. a. Kahn antigen
   b. 0.9 per cent NaCl solution
II. A reliable nonluetic serum (Kahn negative)

The antigen is thoroughly mixed with the salt solution to the given titer and left to ripen for at least ten minutes at room temperature.

PROCEDURE

Pour 0.1 c.c. of serum (reagent II) in each of two small test tubes and inactivate it at 56° C. for twenty minutes, together with a little more than

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1.5 c.c. of saline solution. Remove tubes from water bath and cool to room temperature. Add 0.01 c.c. of the ripened antigen (1) to each of the serum tubes and shake by hand or shaker for three minutes. Add 0.5 c.c. of spinal fluid to one tube and 1.0 c.c. to the other, and again shake for three minutes. The reaction is now read in the usual way. If a precipitate has formed only in the tube containing 1.0 c.c. of spinal fluid, the reaction is weakly positive; if in both tubes, it is considered strongly positive. If only small quantities of spinal fluid are at disposal, the test may be made with one tube and 0.75 c.c. of spinal fluid. To make the reaction more clearly visible, it is advisable to centrifuge at a high speed for about ten minutes. If the reaction is positive, a precipitate on the bottom of the test tube is seen, the supernatant fluid being transparent. After shaking the test tube, the flocculation is distinctly visible. In case of a negative reaction the fluid remains turbid and there is no precipitate. For measuring accurately the small amount of antigen, we use a 0.200 c.c. serologic pipette with a bent tip (Fig. 1), which enables us to direct the fluid to one spot. By using this pipette, dispersion and loss of substance on the wall of the test tube are prevented.

Fig. 1.

In order to prove that the spinal fluid itself does not affect or modify the reaction, we added to 4 test tubes containing 0.1 c.c. of inactivated Kahn positive serum, the usual quantity of antigen. After shaking the tubes, we added to two, 0.5 c.c. and 1.0 c.c. of negative spinal fluid, and to the other, two equal quantities of salt solution. The reaction in both cases was the same in character and in degree. Negative sera treated with negative spinal fluid never gave positive results.

A serologic reaction for gonococcus and echinococcus antibodies, based on the same principle, will be reported shortly.

AN EFFECTIVE METHOD FOR ANESTHETIZING SMALL ANIMALS*

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IN THE laboratory investigation of clinical problems in which small animals are utilized, the problems of struggling and restraint are present. The difficulties arise particularly in carrying out ether anesthesia without adequate assistance upon animals such as the cat.

One can readily work alone without difficulty by placing not too docile animals within a wooden box. If the dimensions of length, height, and width, respectively, are about 16 × 13 × 11 inches, it adequately accommodates any cat or smaller animal. Windows should be inserted in at least two of the sides so that the animal's condition is observable. A hinged cover is constructed on top of the box.

An ether vaporizer to be used with the box has been devised here. This is illustrated in Fig. 1. It is constructed quickly and easily with little expense, and its parts are obtainable.

The ether is contained in a closed separatory funnel, and is allowed to run through the stopcock into the vaporizing flask when needed. The flask must be Pyrex and widemouthed, a convenient capacity being 500 c.c. It must be fitted with a rubber stopper. Erlenmeyer flasks of this capacity, which take a No. 10 stopper, are manufactured by Corning Glass Works, Corning, N. Y. An eight-ounce widemouthed nursing bottle with a No. 10 stopper may be substituted, but it will not take as much ether. Two wires are each inserted into a glass tube, and the tube at its two ends is flame-sealed around the wire. These shielded wires are inserted through the stopper to extend down into the flask. Near the bottom, each wire leaves the glass shield and is firmly soldered or welded to a broad flat copper electrode; the two electrodes are kept carefully separated to prevent sparking (which might explode the ether). Above the flask, the wires are extended upward by well-insulated cord through a switch to a plug. This may be inserted into any socket carrying alternating current (110 to 220 volts). The switch must be well-enclosed, by tape or other means, to prevent the chance of open sparking.

An aqueous copper sulfate solution is poured inside the flask. Its level must be kept well *above* the electrodes. If the solution is not too concentrated (a pale blue color indicates the proper saturation), its electrical conductivity is small; and thus when the current is applied, its resistance will create heat. This rapidly warms the ether to its boiling point (about 35° C.), and vaporizes it. The vapor is conducted away from the flask in a steady stream through glass and rubber pressure tubing to the animal box. A terminal glass nozzle should penetrate the box through a fitted hole bored at a low level to allow the ether vapors to rise and permeate the whole interior.

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Within the vaporizing flask the volume of ether is always observable, since being light and immiscible with water it rests as a distinct layer above the blue solution. There is no danger of its diffusing downward to the electrode area. Sparking between the electrodes is further minimized by using an alternating current. This alternation also prevents appreciable solution of copper, which would erode the electrodes and make the fluid muddy.

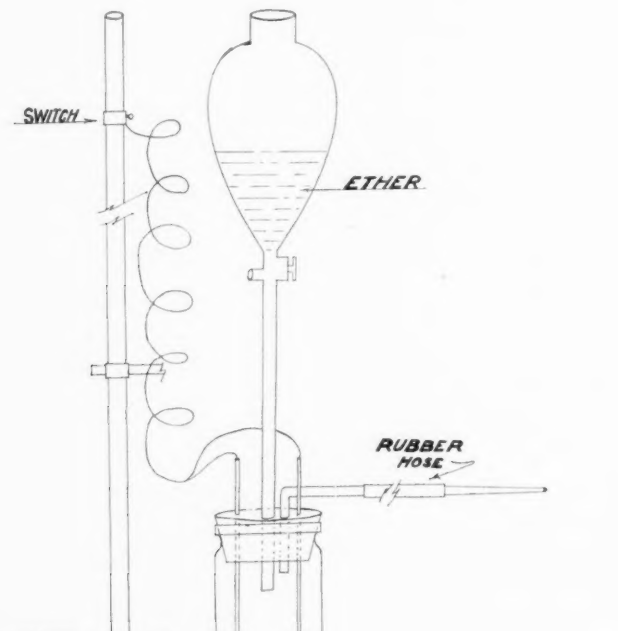

Fig. 1.

We use this apparatus frequently and can state that with ordinary care it is no more dangerous than any other laboratory appliance, although it is unquestionably hazardous in the hands of inexperienced persons. It is quick and sparing of ether. By observation of the ether box, the stages of anesthesia are clearly followed. In the surgical stage the animal may be removed and tied down to an animal board. It is kept anesthetized thereafter by applying a cone to its nose.

CHEMICAL

SERUM BILIRUBIN: A PROCEDURE FOR THE DETERMINATION OF INDIRECT AND DIRECT VALUES

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THE original van den Bergh technique and the modification to this method introduced by Thannhauser and Andersen have been used extensively for the determination of serum bilirubin. Both procedures, however, present several disadvantages which can be summarized as follows: (1) It is difficult to match the color of azorubin with that of artificial standard solutions; (2) loss of bilirubin occurs in the protein precipitate; (3) values of less than 1 mg. cannot be read accurately; (4) separate determinations of the direct and indirect fractions cannot be made since estimation is on total bilirubin; and (5) in the Thannhauser and Andersen method the personal error involved in the correct differentiation of direct and indirect types of reaction is considerable.

The fact that sera in which the bilirubin gives a direct reaction also contain a varying amount of indirect reacting bilirubin is theoretically of interest. It has been postulated that the proportion between these two types of bilirubins is different in various types of jaundice, and therefore their separate determination should give diagnostic and prognostic information. Consequently, several techniques have been devised to measure separately the quantity of direct and indirect fractions of total bilirubin; furthermore, new techniques have been devised which avoid the deficiencies of the former methods and provide for the accurate determination of bilirubin. In this paper a technique for determination of bilirubin is described which is based in part on two methods previously outlined.

EARLIER METHODS FOR MEASURING BILIRUBIN

Varela Fuentes[1, 2] developed a technique on the basis of the solubility of indirect reacting bilirubin in chloroform. In his procedure the indirect reacting bilirubin. Therefore we subtract from the final reading of direct bilirubin this After evaporation of the chloroform the dry residue is redissolved in chloroform and alcohol and then diazotized. The concentration of direct bilirubin in the same sample of serum is determined by adding diazo reagent and alcohol after extraction of the indirect bilirubin with chloroform. The precipitate is separated by filtration, and the reading is made on the filtrate by means of a standard colorimeter.

The technique for the extraction of indirect reacting bilirubin proposed by Varela Fuentes appeared to us to be the most efficient method thus far reported.

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We wish to express our appreciation to Miss Elizabeth Maclay for technical assistance.
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It has been used in the work to be described in this paper with slight modifications which were made to adapt it to the photelometer.

Several objections can be raised to the use of the method of Varela Fuentes for the determination of direct bilirubin; essentially it has the same disadvantages as the original van den Bergh method and the Thannhauser and Andersen technique, that is, loss of bilirubin in the protein precipitate and the difficulties of matching color with that of artificial standard solution.

One of us (A. E. O.)[3] modified the technique for determination of bilirubin proposed by van den Bergh and Grotepass[4] in order to adapt it for reading in the photelometer. Their procedure for determination of direct reacting bilirubin is based on the fact, known since the first publications of van den Bergh, that this fraction couples immediately with the diazo reagent in the serum; the addition of 96 per cent alcohol is unnecessary for the reaction. In their technique 50 per cent buffer alcohol (final concentration in tube, 25 per cent) is used to maintain a constant pH in the tubes for the direct and indirect reaction. The use of alcohol, however, even in such a low concentration, is a cause of error, for it allows part of the indirect bilirubin to react and thus increases the color of the direct reaction. This fact has been mentioned previously by Malloy and Evelyn[5] and has been confirmed in the present investigation. The buffer alcohol is not necessary when the photelometer is used, since the differences in pH do not exert any influence on the reading. In the determination of indirect bilirubin the method obviously can be applied only in those cases in which the serum contains this fraction alone, as in certain cases of hemolytic icterus. When both fractions are present, as usually occurs in obstructive and parenchymatous jaundice, the method really determines total bilirubin. Even so, the estimation is incomplete, since as mentioned before some direct bilirubin is carried out in the protein precipitate.

Malloy and Evelyn developed a procedure to make separate determinations of direct and total bilirubin. A considerable improvement is made by the use of 50 per cent alcohol in the measurement of total bilirubin; Malloy and Evelyn have demonstrated that complete coupling of the bilirubin takes place in the presence of 50 per cent alcohol without any precipitation of protein. The estimation of direct bilirubin is based on the same principle we have used, namely, the ability of this fraction to react in water solution; the reading is also made in a photelometer. Later some differences between the two methods will be mentioned.

DESCRIPTION OF PRESENT TECHNIQUE

Qualitative Test.—The qualitative test is first performed on a sample of serum as follows. To 1 c.c. of serum, generally diluted, 2 c.c. of diazo reagent is added. Three well-known results may be obtained:

1. A direct reaction may occur. The color change then is detected as soon as serum and diazo reagent are mixed together; it reaches its maximal intensity in from sixty to ninety seconds. The color is pink in undiluted serum and purplish in diluted serum.

2. An indirect (delayed or negative direct) reaction may occur. No change of color then is noted during the first two minutes after addition of the diazo

reagent. Between four and ten minutes after the diazo reagent is added a golden color appears, in about thirty minutes a brownish color, and after one to one and a half hours the characteristic pink color develops. This color is similar to that of the direct reaction in the undiluted serum.

3. A biphasic, direct reaction may occur. This type of reaction has been found only three times in more than 400 cases observed by us. The color which appears during the first minute is not a typical pink, but a brownish red which generally reaches its maximal intensity in two or three minutes after the diazo reagent is added.

Quantitative Test.—After the qualitative test, quantitative determination of direct and indirect reacting bilirubins is made.

Direct Reacting Bilirubin.—The quantitative determination of direct reacting bilirubin is carried out only if either a direct or biphasic reaction has occurred in the qualitative test. The volume of the serum and diazo reagent is made up to 10 c.c. with distilled water, and the photelometer reading is made during the first ten minutes after the water is added.

In spite of the fact that a filter having a maximal transmission at 530 to 535 millimicrons is used, the color of the serum itself influences the reading. By making a control series of readings with normal sera against water "blank," we have found an average photelometer reading of 96 which equals 0.4 mg. of bilirubin. Therefore we subtract from the final reading of direct bilirubin this correction factor of 0.4 mg. This procedure is accurate and avoids the preparation of a normal "serum blank" for every determination.

Indirect Reacting Bilirubin.—To another 1 c.c. portion of the serum in a centrifuge tube marked at 10 c.c. is added 2 c.c. of sodium sulfate (12 per cent solution prepared from anhydride), and the solution is mixed. To this is added 5 c.c. (or more) of chloroform. The tube is shaken vigorously for thirty seconds, then centrifuged for about five minutes at high speed. The yellow chloroform extract remains under the turbid serum. The capillary end of a pipet is introduced to the bottom of the tube and almost all the chloroform is aspirated. A slanting mirror should be placed under the tube so the operation may be watched. The aspirated chloroform is transferred to a test tube of at least 2 cm. in diameter. Extraction of the serum is repeated with a new portion of chloroform and the second extract is added to the first. Extraction must be repeated until the chloroform is colorless. Usually three to four extractions are sufficient.

The combined chloroform extracts in the test tube are evaporated in a water bath at 80° C. The dry adherent residue is taken up in 0.5 c.c. of chloroform and 2 c.c. of 96 per cent alcohol, which dissolve the bilirubin. To this is added 0.5 c.c. of a fresh diazo reagent (final volume 3 c.c.). The red color of azobilirubin does not develop completely until after ten minutes. The reading is made in the photelometer against water blank.

We have used the Cenco Sheard-Sanford photelometer equipped with a Cenco No. 2 filter which has a transmission range of 550 to 700 millimicrons. The calibration curve is obtained by the following procedure: Five milligrams of purified bilirubin is dissolved in 100 c.c. of chloroform, and 10 c.c. of this solution is evaporated under carbon dioxide on a water bath, until it is nearly dry. To the residue is added 80 c.c. of a solution consisting of 50 per cent alcohol which contains 10 c.c. of a buffer solution of pH 6.6 to every 100 c.c. The buffer alcohol solution is heated for some time in order to evaporate the chloro-

form. Next, the solution is brought to room temperature and to it is added 20 c.c. of the reagent consisting of Solution A, 1 Gm. sulfanilic acid, 15 c.c. of 25 per cent hydrochloric acid with distilled water up to 1 liter, and Solution B, 0.5 per cent sodium nitrite in aqueous solution. Ten cubic centimeters of Solution A is mixed with 0.3 c.c. of Solution B shortly before use. Additional buffer containing alcohol is added to the solution after the reagent and the total quantity has been brought up to 100 c.c. The pigmented solution which is to be examined must be left in the dark for fifteen minutes in order to complete the coupling.

The concentration of bilirubin as its azo derivative equals 1/200,000 or 0.5 mg. in 100 c.c. This corresponds to a concentration of bilirubin of 5 mg. in each 100 c.c. of serum when the analysis is carried out on 1 c.c. of serum and the final volume equals 10 c.c.

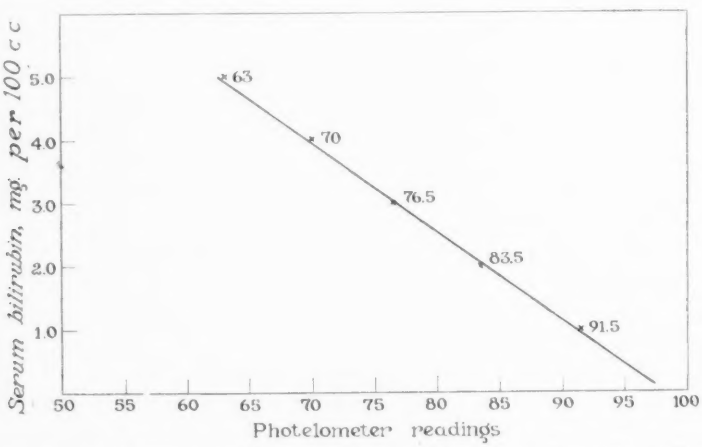

Fig. 1.—Curve of photelometer readings showing relationship with concentration of serum bilirubin.

Other points on the curve are obtained in a similar manner using volumes of the original solution of bilirubin of 8 c.c., 6 c.c., 4 c.c., and 2 c.c. By this procedure, as is shown in Fig. 1, there is obtained, for practical purposes, a straight line relationship* up to values corresponding to a concentration of 3 mg. of bilirubin in each 100 c.c. of serum. If higher concentrations than this value are obtained, it is necessary to dilute the serum previous to the determination in order to obtain a value of less than 3 mg. in each 100 c.c. Appropriate factors are then applied to obtain the final value of bilirubin. This procedure is adopted because of the fact that when values of more than 3 mg. per 100 c.c. are subjected to the diazo reaction, the photelometric readings depart too greatly from the assumed straight line relationship. Also, the concentration of the compound produces a dark color and turbidity may develop.

COMMENT

Direct Reacting Bilirubin.—We have found that in serum diluted before the addition of diazo reagent the reading of direct bilirubin yields higher figures

*It is known that, in the case of solutions of substances which possess characteristic absorption zones, the concentration (C) is proportional to the negative logarithm of the transmitted light (I); that is, $C = -k \log I$. For the range of dilutions used in these investigations, however, departures from the linear relationship as shown in Fig. 1 and the values obtained from the semilogarithmic plot are not considered as being significant.

than in undiluted serum. Serum diluted after coupling behaves like undiluted serum. The higher the concentration of pigment, the greater the difference in the results. A curve of the quantities obtained at different dilutions can be drawn demonstrating that relationship, until a limit of dilution is reached, after which no further variation occurs (Table I).

These differences were first attributed to variations in the color of the reaction, owing to changes in pH, the azorubin in undiluted serum being red-pink and purplish or red-violet in diluted serum. In the first case the proteins and other buffer mechanisms present in serum neutralize partially the acid diazo reagent giving a final reaction that is weakly acid. In the diluted serum this does not occur and a lower pH results. Griffiths and Kaye[6] have mentioned the diverse tones of color present according to the variations in pH. In the experiment described in Table I, however, we found that these differences in color did not exert an influence on the reading; also that the final results were comparable when several different fluids were used to make the dilutions.

TABLE I

READINGS ON SERUM DILUTED BEFORE AND AFTER COUPLING

	BILIRUBIN, MG. PER 100 C.C. AFTER USE OF DILUTIONS OF		
	1:10	1:20	1:40*
A. Serum diluted after coupling	22.6	24.0	24.0
B. Serum diluted before coupling			
Dilution with serum of normal dog†	26.6	32.6	37.6
Dilution with diazo reagent	24.6	27.6	31.6
Dilution with water	27.6	31.6	35.6

*Dilutions greater than 1:40 gave same results.
†The serum of a normal dog contained 0.05 mg. per 100 c.c. of indirect bilirubin.

The higher readings obtained in serum diluted before coupling may be explained as follows: When the concentration of direct bilirubin is high, the diazo reagent is unable to cause all the bilirubin to react; consequently a variable amount remains undetermined. In diluted serum less pigment is present, and with the same quantity of diazo reagent the combination is complete and the total quantity of direct bilirubin present is determined. The proper dilution for serum in a case of jaundice can be judged with relative certainty by the intensity of the yellow color of the serum. We usually make a 1:5 or 1:10 dilution. After diazotization has taken place, the reading is made in the photelometer; if the reading is below the equivalent of 3 mg. (above 75 on the scale), the dilution is correct and the calculation can be made therefrom in milligrams of bilirubin. When the reading is higher than 3 mg. (below 75 on the scale), the dilution is increased. With diluted serum we have found that the most accurate results are obtained if the readings are kept between 1 and 3 mg. (75 to 90 on the scale). The disadvantage of repetition of the reading is necessary only in case of unknown sera and with practice it can usually be avoided.

We do the reading of direct bilirubin the first ten minutes after the water is added, because after this time the indirect bilirubin also takes part in the reaction. It is well known that the indirect bilirubin can react slowly in water solution, and this is evidenced by the fact that in sera in cases of hemolytic jaundice the development of color starts usually after ten minutes. When both

fractions are present, we have observed differences of several milligrams between the readings at five and thirty minutes. This is especially true when the amount of indirect bilirubin is high.

Indirect Reacting Bilirubin.—Grunenberg[7] first noted the different behavior with chloroform of direct and indirect reacting bilirubins and demonstrated that the pigment in serum from a patient who had hemolytic jaundice was soluble in chloroform while that from a patient who had obstructive jaundice was not. Andrewes,[8] Collinson and Fowweather,[9] Roberts[10] and Newman[11] have confirmed these results. Hunter[12] included the different solubilities in chloroform in a summary of the properties of direct and indirect reacting bilirubins.

De Castro[13] presented a method for the determination of both types of bilirubin in which the indirect type of bilirubin was extracted from the serum with chloroform. Varela Fuentes showed that with this technique, when 2 c.c. of chloroform were used and the extraction was done only once, 50 per cent of the indirect reacting bilirubin remained in the serum. Consequently he modified the method so that 5 c.c. of chloroform were used and several extractions were made. In most cases extraction by this method was complete, but when the concentration of bilirubin was high, a small portion of the pigment remained in the serum. With the use of the 12 per cent solution of sodium sulfate, extraction was improved and the chloroform removed practically all the indirect bilirubin.

Before adopting the technique of Varela Fuentes it was necessary for us to confirm that: (1) chloroform extracts only indirect reacting bilirubin without affecting the direct type, and (2) chloroform extracts the indirect reacting bilirubin completely. This was done as follows:

In serum in which both direct and indirect reacting bilirubins were present, the direct bilirubin was measured by the method recommended in this paper. In a different sample of the same serum complete extraction of the indirect bilirubin with chloroform was carried out. After extraction, a mixture of serum, sodium sulfate, and chloroform residues remained in the tube. After centrifuging, the three layers separated: the upper layer was a yellow liquid (mostly sodium sulfate in which was dissolved part of the direct bilirubin); the middle layer was the protein precipitate which carried the remainder of the direct reacting bilirubin, and the bottom layer consisted of a small quantity of colorless chloroform. The upper layer was poured into another tube and the chloroform was evaporated in the presence of carbon dioxide to prevent oxidation of the bilirubin. The dry precipitate was dissolved in 10 to 20 c.c. of 5 per cent urea solution. Usually all the precipitate went into solution but sometimes a portion remained insoluble. To this solution was added the yellow upper layer mentioned and 1 c.c. of the mixture was diazotized. A direct reaction was obtained and the reading was made in the photelometer. In a good proportion of cases approximate agreement was found between the values for direct bilirubin and those of bilirubin recovered from serum after extraction with chloroform (Table II). Cases in which recovery of direct bilirubin was not complete were those in which a portion of the precipitate containing some bilirubin was insoluble. Apparently this fact accounts for the incomplete recovery of direct reacting bilirubin in these cases.

It is easy to observe that after several extractions the original yellow serum of hemolytic jaundice turns a whitish color. On the other hand, the serum of ob-

structive jaundice remains yellow no matter how many extractions are made with chloroform. A more definite proof of the complete extraction of bilirubin with chloroform was obtained by the following method: Three c.c. of 96 per cent alcohol were added to serum from a patient who had hemolytic jaundice after complete extraction with chloroform. The mixture was shaken and centrifuged. The supernatant fluid was pipetted off and diazotized. In a great majority of cases no color developed; its absence indicated the absence of bilirubin in the serum. We found that the only time at which a small portion was not extracted was in the presence of hemolytic jaundice, when the concentration of bilirubin in the serum was high. This portion was never more than 15 per cent of the whole amount of indirect reacting bilirubin present. Therefore, from a practical standpoint, it may be considered that chloroform produces a complete extraction of indirect reacting bilirubin. In this point we agree with the results reported by Varela Fuentes.

TABLE II

VALUES FOR DIRECT REACTING BILIRUBIN IN SERUM BEFORE AND AFTER EXTRACTION OF INDIRECT BILIRUBIN WITH CHLOROFORM

DIRECT BILIRUBIN		INDIRECT BILIRUBIN
BEFORE EXTRACTION	AFTER EXTRACTION	
13.8	14.4	3.9
14.1	13.2	2.5
44.6	43.7	3.7
27.5	28.0	2.7
16.8	17.0	2.7
22.6	23.0	2.4

TABLE III

COMPARISON BETWEEN NEW METHOD AND THE THANNHAUSER AND ANDERSEN METHOD IN HEMOLYTIC PROCESSES

DIAGNOSIS	NEW METHOD		THANNHAUSER AND ANDERSEN METHOD	
	DIRECT BILIRUBIN	INDIRECT BILIRUBIN, MG. PER 100 C.C.	REACTION	MG. PER 100 C.C.
Congenital hemolytic icterus	0	3.2	Indirect	2.6
Congenital hemolytic icterus	0	10.0	Direct	9.5
Congenital hemolytic icterus	0	2.5	Indirect	2.9
Congenital hemolytic icterus	0	1.3	Indirect	1.6
Acquired hemolytic icterus	0	2.8	Direct	4.1
Subacute hemolytic anemia	0	1.5	Direct	2.4
Acquired hemolytic anemia	0	5.4	Direct	5.8
Acute hemolytic icterus	0	9.6	Direct	10.7

COMPARISON OF NEW METHOD WITH OTHER METHODS

Thannhauser and Andersen Procedure.—Qualitative reaction.—The qualitative results obtained with the new technique and with the Thannhauser and Andersen technique in a group of cases of hemolytic processes are listed in Table III. In five cases the results with the Thannhauser and Andersen technique were incorrect. Apparently the short period of time, two minutes, between the addition of the diazo reagent and the reading of the qualitative test used in the Thannhauser and Andersen method accounts for the wrong reports. When the concentration of indirect bilirubin is high, a change of color starts at the

end of two minutes; this color is never the characteristic pink or red violet of direct bilirubin but may lead to misinterpretation.

Quantitative Reaction.—The quantitative results from the same two techniques are shown in Fig. 2. In every case the values of total bilirubin were higher with the new technique. The higher the quantity of bilirubin present the greater was the difference in the readings given by the two methods. Occasionally the concentration was found to be as much as three times as high with the new technique.

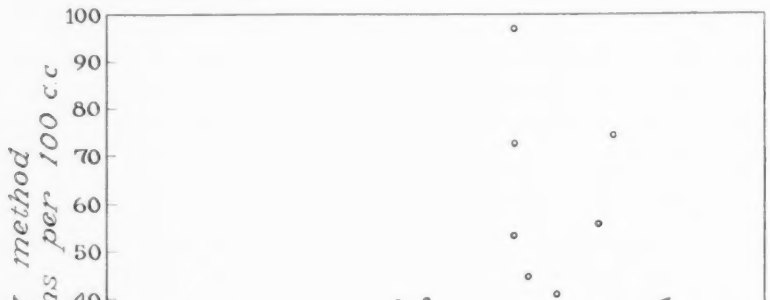

Fig. 2.—Determination of bilirubin in 65 cases of intrahepatic and obstructive jaundice. Quantitative results.

TABLE IV

COMPARISON OF MALLOY AND EVELYN TECHNIQUE AND THE NEW TECHNIQUE: QUANTITATIVE AND QUALITATIVE RESULTS

CASE	BILIRUBIN, MG. PER 100 C.C.				
	MALLOY-EVELYN TECHNIQUE		NEW TECHNIQUE		TOTAL
	DIRECT	TOTAL	DIRECT	INDIRECT	
1	1.0	2.5	Negative	2.5	—
2	0.3	0.8	Negative	0.3	—
3	0.7	1.2	Negative	0.8	—
4	1.2	1.5	1.5	0.3	1.8
5	5.0	7.4	6.5	0.2	6.7
6	10.0	13.5	13.6	1.1	14.7
7	7.5	10.4	10.6	1.0	11.6
8	0.7	1.7	2.6	0.6	3.2

Malloy and Evelyn Technique.—In Table IV the results of the new technique and the Malloy-Evelyn technique are compared. In the latter, determinations are made of the direct reacting bilirubin and of total bilirubin. Quantitative results for total bilirubin were similar, and from a practical standpoint both methods apparently are equally reliable. The qualitative results disagreed, however, because in several cases "direct" reacting bilirubin was found with

the Malloy-Evelyn technique whereas it was not found with the new technique. The explanation for this apparent contradiction is as follows: In the Malloy and Evelyn technique readings for direct bilirubin are always made regardless of the qualitative type of reaction. In the new technique, readings for direct bilirubin are made only when the qualitative reaction gives evidence of the presence of direct bilirubin. As has been stated, indirect bilirubin can react slowly in water solution; consequently, if a reading is made thirty minutes after the diazo reagent has been added to a serum from a normal person or to one from a patient who has hemolytic jaundice, the coupling is advanced enough to give a false direct reaction. This ability of the indirect bilirubin to react slowly in water solution explains, in our opinion, the results reported by Cantarow, Wirts, and Hollander,[14] who used the Malloy and Evelyn technique. These writers made the reading at the end of thirty minutes and found what they considered direct reacting bilirubin in the serum of normal persons, in cases of hemolytic jaundice, and in cases of hepatic and biliary disturbances; the values for total bilirubin, however, were within normal limits. Furthermore, they found that in some cases of hepatic and biliary disease the qualitative test gave a negative direct result while the quantitative procedure indicated the presence of "direct" bilirubin. This apparent contradiction is due to the fact that in the period of time which elapsed before the reading was made some of the indirect bilirubin was changed to azorubin. It is true that those results can be taken as a conventional term and probably the values would be comparable in the same group of disorders. We think it preferable, however, to establish a clear distinction between the qualitative reactions and evaluate the direct bilirubin only when its presence has been proved by an immediate reaction.

SUMMARY

A technique for the determination of direct and indirect reacting bilirubin present in serum is described. This procedure is a combination of two previously described methods which have been somewhat modified. A comparison has been made between values obtained with the modified procedure and those obtained with such standard methods as the Thannhauser-Andersen modification of the van den Bergh method and the Evelyn and Malloy procedure. Evidence is presented which demonstrates the difference in solubility of the direct and indirect reacting types of bilirubin and it is shown that this difference allows their separate determination. The ratios in which the two types occur are also given. The clinical importance of these two types of bilirubin is to be presented in another paper.

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STUDIES OF PANCREATIC FUNCTION*

I. The Determination of the Lipidolytic Enzymes of Blood Serum

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THE DIAGNOSIS of pancreatic disease, other than diabetes mellitus, is difficult, a situation due in part to the limitations of laboratory methods. The laboratory approach to this problem has been along two general pathways: (1) the determination of enzymes of pancreatic origin (amylase, lipase) in the blood serum; (2) the estimation of the amount, bicarbonate content, and enzymes of the external secretion of the pancreas following pancreatic stimulation.

In acute inflammatory lesions of the pancreas, where either a disintegration of pancreatic tissue or obstruction in the ducts causes the back-passage of abnormal quantities of pancreatic enzymes into the blood stream, the determination of the amylase or lipase content of the blood serum has been found very useful. In those lesions of the pancreas where there is a decrease in the amount of enzymes secreted, however, the information obtainable from blood studies is without significance. The indications of an elevation of pancreatic enzymes in the blood serum, in the presence of the other clinical findings, are clear, but the observation in the serum of a lower than normal content of enzymes, which have characteristics similar to those secreted by the pancreas, is difficult to interpret because these enzymes have an extrapancreatic, as well as pancreatic, origin.

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In those pancreatic diseases which are characterized by a deficient external secretion, the clinician obtains some information by observations of the stools. Bulky stools, containing undigested muscle fibers, starch, and increased fat, suggest a deficiency in pancreatic secretion. Pratt[1] has stressed the importance of a gross inspection of the stools, and he calls attention to the rather specific relation of creatorrhea to pancreatic insufficiency.

Lagerlöff[2] states that the determination of the enzyme content of the stools is worthless. According to this author, lipase is inactivated in the feces. Moreover, amylase originates in the saliva as well as in the pancreatic juice.

Recently, important advances have been made in the development of laboratory procedures for the direct testing of pancreatic function. Ågren and Lagerlöff[3] appear to be the first investigators to have developed a successful technique for collecting pancreatic juice uncontaminated with saliva or gastric juice. In 1936 these authors described a procedure in which a double lumen tube is introduced into the upper alimentary canal and the contents of the stomach and duodenum are removed simultaneously by suction, before and after the intravenous administration of secretin. Ågren and Lagerlöff, also Diamond and Siegel[4] in this country, have reported that the secretin test gives much valuable information concerning disturbed function of the pancreas, particularly in chronic states.

A new method for evaluating pancreatic function has recently been reported by Free, Beams, and Myers.[5] In this procedure a Rehfuss or Einhorn duodenal tube is introduced into the stomach and the gastric contents are removed. The tube is then passed into the duodenum and a twenty-minute fasting sample of duodenal contents is collected. Twenty-five c.c. of olive oil are then injected slowly through the tube, and after ten minutes siphonage is started again. Three twenty-minute samples of duodenal juice are collected. All samples of duodenal juice are examined for total volume, proteinase, amylase, lipase, and icteric index. Data were obtained upon patients with pancreatic disease which showed marked departures from normal values. These authors report results as good by the use of a single duodenal tube as with a double lumen tube. Exclusion of gastric juice results from the inhibitory effect of olive oil on gastric secretion and motility. This test appears to be on a sound basis and has much in its favor in the way of simplicity and practicality of procedure.

Numerous methods for the determination of lipidolytic enzymes have been proposed. These have involved the use of titration, stalagmometric, manometric, and electrometric techniques. Of these, representative ones will be discussed here. In early reports in the literature, the fatty acid liberated by the hydrolysis of substrate was titrated with standard alkali. In 1911 Rona and Michaelis[6] developed a stalagmometric method, using tributyrin as a substrate. In this method the increase in the number of drops of a solution containing enzyme, substrate, and buffer, due to changes in surface tension, is taken as a measure of enzyme activity. Rona and Lasnitzki,[7] in 1924, devised a manometric method for the estimation of lipase. This method measures manometrically the volume of carbon dioxide released from sodium bicarbonate by the action of butyric acid, the amount of butyric acid being dependent upon the concentration of the

lipase, which promoted the hydrolysis of tributyrin. This method has certain advantages: the pH of the solution is readily maintained and one of the end products of the reaction is continuously removed. A titration method for the determination of lipase was published by Willstätter, Waldschmidt-Leitz, and Memmen[8] in 1923. In this procedure the fatty acid liberated by action of the lipase upon a buffered olive oil emulsion is titrated with standard alkali. In 1932 Cherry and Crandall[9] published a modification of the Loevenhart[10] (1907) method for the determination of esterase, in which the fatty acids resulting from the hydrolysis of a buffered ester or fat emulsion by the enzymes of a biologic material are titrated with twentieth-normal sodium hydroxide. These investigators reported that after ligation of the pancreatic ducts in dogs, they could demonstrate the presence in serum of an olive oil-splitting enzyme (lipase) which normally did not occur in the blood serum. They also found that the activity of an enzyme normally present in blood, as measured by the hydrolysis of ethyl butyrate or tributyrin (esterase), did not show uniform increases following pancreatic duct ligation. They, therefore, concluded that their test for lipase was specific for pancreatic injury. Recently, Troescher and Norris[11] have proposed a method in which the action of the enzyme upon ethyl butyrate in a phosphate buffer solution is measured by the change in pH of the solution due to the butyric acid liberated.

Our interest in this problem arose from a realization of the limitations of present methods for determining the lipidolytic enzymes of the blood. The serum lipase determination has been reported to be especially useful in the diagnosis of pancreatic lesions of the obstructive type.[12-14] The methods used in this work require a 24-hour incubation of serum with substrate. Obviously a procedure for estimating the concentration of an enzyme which requires twenty-four hours of hydrolysis is undesirable from both a theoretical and practical point of view. It is well known that to make an accurate determination of the concentration of an enzyme, one should measure the initial rate of hydrolysis. Furthermore, when a serum lipase determination is desired, the patient usually is acutely ill and waiting for twenty-four hours on an analysis is very objectionable.

Of the various methods proposed for estimating lipase or esterase action, we believe the most suitable procedure for clinical work is the simple titration of the fatty acid liberated by enzymic activity upon a suitable substrate. In attempting to develop a method which would be more accurate and sensitive our attention was directed to a consideration of the following conditions: (1) the substrate used; (2) the emulsifying agent; (3) the pH at which the test is run and therefore the buffers used; (4) removal of the end products of the reaction; (5) the temperature at which the test is carried out; (6) the method of stopping the enzymatic hydrolysis. Our results and conclusions with respect to each of these conditions are set forth below.

SELECTION OF SUBSTRATE

Cherry and Crandall[9] studied the activity of pancreatic and liver extracts and blood serum upon 21 different esters, with particular emphasis upon ethyl butyrate, tributyrin, and olive oil. These investigators abandoned the use of

ethyl butyrate and tributyrin on the grounds that the latter substances were split by blood esterase, whereas only blood lipase splits olive oil. Other investigators, however, have used ethyl butyrate and tributyrin on the same basis as olive oil. Two other new and possible substrates, benzyl butyrate and benzyl stearate, were suggested by the work of Balls and Matlack,[15] who found that the esters of benzyl alcohol are split more rapidly than those of other alcohols. Benzyl stearate was eliminated from use because at ordinary temperatures it is a solid and is extremely difficult to emulsify. The five substrates selected for study were ethyl butyrate, benzyl butyrate, tributyrin, olive oil, and triolein. All five of these exist in the liquid form at room temperatures and are fairly easy to emulsify; two are simple esters and three are glycerides, a selection which appeared to offer an opportunity to study the question of whether the biologic fluid being examined contains an esterase, a lipase, or both. Triolein was selected in order to have a pure triglyceride of a known long chain fatty acid for comparison with olive oil, an impure mixture of glycerides and other substances.

TABLE I

RATE OF SPLITTING OF DIFFERENT SUBSTRATES

SUBSTRATE	GLYCEROL EXTRACT OF PANCREAS	GLYCEROL EXTRACT OF LIVER	HUMAN SERUM	CAT SERUM	RABBIT SERUM	DOG SERUM
Ethyl Butyrate (0.25 c.c.)	5.20 (1 hr.)	4.67 (1 hr.)	0.33 (24 hr.)	0.41 (24 hr.)	0.28 (24 hr.)	1.90 (24 hr.)
Tributyrin (0.25 c.c.)	13.40 (1 hr.)	2.68 (1 hr.)	0.83 (1 hr.)	1.01 (1 hr.)	1.19 (3 hr.)	1.95 (1 hr.)
Benzyl Butyrate (0.25 c.c.)	9.77 (1 hr.)	6.52 (1 hr.)	0.49 (3 hr.)	0.65 (3 hr.)	0.53 (3 hr.)	2.19 (3 hr.)
Olive Oil (1.00 c.c.)	6.24 (1 hr.)	00 (24 hr.)	0.18 (24 hr.)	0.26 (24 hr.)	0.22 (24 hr.)	0.38 (24 hr.)
Triolein (0.25 c.c.)			0.15 (24 hr.)	0.31 (24 hr.)		0.33 (24 hr.)

Expressed as c.c. of 0.1 N alcoholic KOH per c.c. of serum or c.c. of tissue-glycerol preparation. Parentheses show amount of substrate in column 1 and time of hydrolysis elsewhere.

The results of this study are recorded in Table I. Our data show that the greatest amount of hydrolysis is obtained with tributyrin as a substrate in experiments with glycerol extract of pancreas and with human, cat, and rabbit serum; whereas with glycerol extract of liver, benzyl butyrate gave a higher value. These results suggest that tributyrin should be the substrate of choice to obtain the most sensitive test when analyzing for enzymes of pancreatic origin and that benzyl butyrate would be the most sensitive substrate for enzymes of liver origin.

THE EMULSIFYING AGENT

For emulsifying agents a number of substances have been used for measurement of lipidase action. Cherry and Crandall[9] favored acacia; Willstätter, Waldschmidt-Leitz, and Memmen[8] used albumin; and Balls, Matlack, and Tucker[16] have utilized a glycerol solution of ox bile. We carried out a series of estimations to determine the most effective emulsifying agent. The data of

Table II show that the bile-glycerol mixture, prepared according to Balls, Matlack, and Tucker,[16] is the most favorable emulsifying agent when benzyl butyrate is used as substrate; in view of these results it was decided to use bile as the emulsifying agent in our experiments.

THE pH AND SELECTION OF BUFFERS

Balls, Matlack, and Tucker[16] observed that pancreatic lipase does not appear to have a very sharp pH optimum. Experiments with tristearin as substrate and NH_3—NH_4Cl mixture as buffer at pH 8.2 to 8.4 and 7.2 to 7.5 showed very little difference in the rates of hydrolysis. Willstätter and Waldschmidt-Leitz[17] found the optimum pH values for pancreatic lipase were 9.2 and 8.3 with olive oil and tributyrin, respectively, as the substrates. With tripropionin as substrate, Weinstein and Wynne[18] found the optimum pH is 7.2 with phosphate-borate buffer and 9.3 with a glycine buffer. Anrep, Lush, and Palmer,[19] using a phosphate buffer and glycerol triacetate as substrate, began their hydrolysis at a pH of 7.8 and permitted a shift in pH to 7.0. They considered a pH below 7.0 to be too low for satisfactory quantitative studies with lipase.

TABLE II

EMULSIFYING POWER OF DIFFERENT EMULSIFYING AGENTS

SUBSTRATE	BILE (DIFCO)	NA. TAURO-CHOLATE (MERCK)	NA. TAURO-CHOLATE (PFAN-STIEHL)	NA. GLYCO-CHOLATE (PFAN-STIEHL)	FRESH EGG ALBUMIN SOLUTION	DRIED EGG ALBUMIN SOLUTION	5 PER CENT DUPONOL SOLUTION	5 PER CENT ACACIA SOLUTION
Benzyl Butyrate (0.240 Gm.)	11.01	5.75	8.56	5.60	1.99	2.55	0.43	3.21

Expressed as c.c. of 0.1 N alcoholic KOH using 5 c.c. of the emulsifying agent and 1 c.c. of glycerol preparation of calf pancreas. Time of hydrolysis was one hour.

From the work discussed above, it appears that one should carry out the hydrolysis at a pH somewhere within the range of 7.0 to 9.2. In view of the observations mentioned, we decided to fix the buffering conditions so that the initial pH is 8.2 to 8.4, and the pH at the conclusion of the hydrolysis is above 7.0. Under the conditions of our procedure, some shifting in pH is permitted, but this is desirable to avoid using excess quantities of buffer which would have an inhibiting effect. The buffer we found most suitable for our work was sodium diethyl barbiturate, and the amount we determined to be most effective was 0.05 Gm. in the final volume of the test used which is 26 c.c.

In the hydrolysis mixture set up for our method 3 c.c. of 0.1 N butyric or oleic acid are required to shift the pH from 8.4 to 7.0. The buffering system in this procedure, therefore, has the capacity to permit optimum buffering with blood sera having concentrations of lipidolytic enzymes up to 300 units per 100 c.c. If concentrations greater than the latter are encountered, the determination may be repeated, using less serum, to obtain greater accuracy.

In Table III are presented data which show the effectiveness of the buffering system we use in holding the pH within a satisfactory range. This is an important feature of our method; the pH at the end of the hydrolysis is still

in the favorable range, unless very high concentrations of enzymes are encountered, in which case a smaller sample of serum is analyzed. In the Cherry and Crandall method, which has received widespread clinical use in this country, a phosphate buffer mixture with a pH of 7.0 is employed. This is at the lower margin of the pH range recommended by other authors for pancreatic lipase; furthermore, the fatty acid liberated during the hydrolysis shifts the pH to a still lower value, resulting in a corresponding decrease in the sensitivity of this test.

TABLE III

CHANGES OF pH DURING HYDROLYSIS

SUBSTRATE	SERUM	TIME OF HYDROLYSIS	CONTROL pH	pH AFTER HYDROLYSIS
Ethyl Butyrate (0.25 c.c.)	Cat	24 hr.	8.20	7.64
Ethyl Butyrate (0.25 c.c.)	Human	24 hr.	8.20	7.95
Tributyrin (0.25 c.c.)	Cat	3 hr.	8.16	6.20
Tributyrin (0.25 c.c.)	Human	1 hr.	8.31	7.90
Benzyl Butyrate (0.25 c.c.)	Cat	3 hr.	8.15	7.55
Benzyl Butyrate (0.25 c.c.)	Human	3 hr.	8.30	8.06
Olive Oil (1.00 c.c.)	Cat	24 hr.	8.15	7.63
Olive Oil (1.00 c.c.)	Human	24 hr.	8.22	8.09

Using 50 mg. of sodium diethylbarbiturate for each substrate in a hydrolysis with 1 c.c. of serum. Measurements made with a glass electrode.

REMOVAL OF THE END PRODUCT OF HYDROLYSIS

Willstätter, Waldschmidt-Leitz, and Memmen,[8] in 1923, instituted the use of calcium chloride to aid the hydrolysis of a fat by lipase. They attributed their favorable results to the formation of an adsorption compound, fat-calcium-albumin-lipase. Balls, Matlack, and Tucker[16] have continued the use of calcium chloride to aid the reaction. These investigators offer the much more reasonable explanation that the calcium ion combines with the fatty acid liberated in the hydrolysis to form an unionized or insoluble calcium salt. This removal of one of the end products prevents the reversal of the reaction and thus promotes a rapid splitting of the substrate. We adopted the use of the calcium ion, but in our work it was found that the chloride ion of calcium chloride, which was released with the formation of the calcium salt of the fatty acid, markedly reduced the pH of the medium, slowing down the reaction. To avoid this untoward effect, several calcium salts of weaker acids were tested. Calcium acetate was selected as the preferred salt to obtain the calcium ion effect of removal of one of the end products of the reaction and, at the same time, to promote good buffering. Balls, Matlack, and Tucker[16] reported that too much calcium chloride is somewhat inhibitory to this reaction. This effect was not related to lowering of the pH, as these authors maintained a fairly constant pH by the addition of ammonia. We found 0.2 Gm. of calcium acetate in 26 c.c. of solution to be the optimum amount for this reaction. This quantity provides for the fatty acid liberated by blood serum extraordinarily rich in lipase and at the same time does not have an inhibitory effect.

INACTIVATING THE ENZYME

Among the various methods of inactivating an enzyme, the two that are most convenient for an analytic method of this type are inactivation by heat and by alcohol. An important point, however, is that the control should be inactivated by the same method as the experimental. In some methods there exists the error of inactivating the control tubes containing the serum by heating and the experimental tubes containing the hydrolysate by addition of alcohol. The error that may occur from inactivating the same serum by the two methods is illustrated by Table IV.

TABLE IV

DIFFERENCE IN TITRATION VALUE OF SERUM ACCORDING TO METHOD OF INACTIVATION

SERUM	SUBSTRATE	METHOD OF INACTIVATION	C.C. OF 0.1 N KOH
Cat 3	----	Heating in Boiling H_2O	1.78
Cat 3	----	Addition of 100 c.c. of Alcohol-ether Mixture	2.25
Cat 2	Benzyl Butyrate	Heating in Boiling H_2O	1.48
Cat 2	Benzyl Butyrate	Addition of 100 c.c. of Alcohol-ether Mixture	2.47

The results of Table IV show the fallacy of inactivating the control tube by one method and the experimental by another method. The discrepancy exhibited in these data will account for a part of the values found in blood sera by the methods which use the two different procedures for inactivation.

Following the work of Balls, Matlack, and Tucker,[16] we have used a mixture of 9 volumes of 95 per cent alcohol and 1 volume of ether to inactivate both our experimental and control tubes. The alcohol, in addition to inactivating the lipase, provides an excellent medium for titration of the fatty acid released by the enzymatic hydrolysis, suppressing the dissociation of the hydroxyl ions in the mixture, and thus permitting the titration of the fatty acid.

TEMPERATURE AND INDICATOR

Balls, Matlack, and Tucker[16] have found that the optimum temperature for lipase action is around 40 to 50° C. We selected the temperature of 37° C. for our procedure because most laboratories usually keep a water bath at that temperature.

The indicator chosen for this work was phenolphthalein, since this indicator is suitable for titration of a weak acid with a strong base. It is of interest that the end point of this titration, as measured by the glass electrode, is at a pH around 10.2, which is higher on the pH scale than in an aqueous titration with phenolphthalein.

PROPOSED METHOD FOR LIPASE OR ESTERASE DETERMINATION

Reagents

1. *Glycerol-bile emulsifying reagent.*—(Balls, Matlack, and Tucker.) To 50 Gm. of dried oxbile (Bacto-oxgall, Difco Laboratories) in a liter Erlenmeyer flask add 50 c.c. of water. Heat this solution in an autoclave at 15 pounds pressure for two hours. Cool, add 500 c.c. of glycerol, and heat the mixture on a

steam bath until a clear solution is obtained. Keep in a stoppered flask in the ice box. When used, the flask is warmed in a water bath to 60 to 70° C. to facilitate pipetting.

2. *Buffer.*—Dissolve 5 Gm. of sodium diethylbarbiturate in 1 liter of solution.

3. *Calcium acetate solution.*—Dissolve 20 Gm. of calcium acetate, chemically pure $Ca(CH_3COO)_2 \cdot H_2O$, in 1 liter of solution.

4. *Alcohol-ether inactivating mixture.*—To 900 c.c. of 95 per cent ethyl alcohol add 100 c.c. of ether.

5. *Standard alkali.*—A 0.1 N alcoholic solution of potassium hydroxide is used.

Procedure

The Experimental.—Pipette the required amount of the substrate (0.25 c.c. of ethyl n-butyrate, tributyrin, benzyl n-butyrate, olive oil, or triolein) into a large eight-inch test tube. Into this tube are pipetted 5 c.c. of the bile-glycerol solution, previously heated in a water bath to 60 to 70° C. for convenience in pipetting. The tube is shaken, and then placed in boiling water for five to ten minutes, after which it is shaken again until the substrate is completely emulsified. After cooling to room temperature, pipette 10 c.c. of calcium acetate (200 mg.) and 10 c.c. of sodium diethylbarbiturate (50 mg.) into the tube. The tube is then placed in a water bath at 37° C., and when the mixture has reached the latter temperature, 1 c.c. of blood serum is pipetted into the tube and mixed thoroughly with the contents by means of a stirring rod. After the appropriate lapse of time (one hour for tributyrin, three hours for benzyl butyrate, twenty-four hours for olive oil, triolein, and ethyl butyrate), pour the contents of the tube into a 250 c.c. Erlenmeyer flask containing 100 c.c. of alcohol-ether mixture. Rinse the empty test tube with the alcohol-ether digestion mixture and pour the rinsings back into the Erlenmeyer flask. Add approximately 1 c.c. of 1 per cent phenolphthalein solution, and titrate with 0.1 N alcoholic potassium hydroxide to the same shade of pink as the blank. It is best to titrate to a deep permanent pink.

The Blank.—The blank tube is prepared and treated in exactly the same way, except that addition of the substrate is omitted. The substrate used should be neutral, and hence a correction for the omission of substrate in the blank is not needed.

Calculation.—Titration of experimental − titration of blank × 100 = units of lipase or esterase per 100 c.c. of serum.

We have redefined the lipase or esterase unit, and in agreement with conventional usage, have expressed results in terms of amount per 100 c.c. of serum. A unit of lipase or esterase is the amount of enzyme that will liberate 1 c.c. of 0.1 N fatty acid from an appropriate substrate at 37° C. in the time allowed (tributyrin, one hour; benzyl butyrate, three hours; ethyl butyrate, olive oil, triolein, twenty-four hours).

DISCUSSION

Using the improved conditions for enzyme activity which have been introduced into the above procedure, we obtain as high a titration in one hour's

hydrolysis of tributyrin by blood serum as is secured by twenty-four hours' hydrolysis of olive oil with the same serum. If tributyrin hydrolysis is as good a measure of the pathology present in pancreatic disease as olive oil hydrolysis, it would be a marked advance to use this one-hour test with tributyrin as substrate. To study this possibility, experiments with animals have been carried out and will be reported in another paper.[20] We may say here that in those experiments which were designed to simulate pancreatic pathology (pancreatic duct ligation, mecholyl stimulation, pancreatectomy), results obtained by the one-hour test with tributyrin as the substrate have reflected the condition present just as characteristically as when a 24-hour hydrolysis of olive oil was carried out. Chloroform poisoning, however, gave a dissociation of results: that is, an increase in the tributyrinase content of the serum without an increase in the values obtained with olive oil or triolein as substrates. Such results raise a question as to whether or not tributyrinase activity of serum might arise from liver injury as well as from pancreatic disease. The only way this question can be answered is by clinical trials. Our clinical studies thus far are too few to justify any conclusions at this time.

Following the above method with tributyrin as the substrate, we determined the lipase in the blood serum of a group of 30 apparently normal medical students. The values obtained ranged from 35 to 112 units per 100 c.c. of serum, showing a normal distribution curve with a peak at about 65 units. Nine of these values were obtained upon bloods collected in the morning about fifteen hours after food intake, and the remaining values are for bloods collected in the afternoon from individuals in the postabsorptive state about six hours. Some variation during the day appears to occur in the lipase content of blood serum, which, however, is not very marked. In our hands duplicate determinations with this method agreed within ±4.5 tributyrinase units per 100 c.c. of serum.

In all of our studies, a more rapid splitting of substrate was obtained with tributyrin than with the other four substrates used, namely, ethyl butyrate, benzyl butyrate, triolein, and olive oil. In explanation of this, it is suggested that the response measured with tributyrin represents the action of more than one enzyme; possibly tributyrin is attacked by "esterase" because of its short chain fatty acids, and at the same time is split by "lipase" since it is a triglyceride.

Since olive oil is a mixture of different glycerides and other substances and its composition may be expected to vary according to its source and methods of preparation, the use of this substrate in estimating "lipase" activity is to be questioned. For this reason we made parallel studies with a highly purified triolein* preparation as a substrate. The results obtained with triolein and olive oil as substrates, however, compare fairly closely, quantitatively, and have a similar significance in the studies made.

With the procedure outlined above, we have consistently found an enzyme in the blood of the normal dog, cat, rabbit, and human being which splits the lipids of olive oil. This observation is not in agreement with the work of Cherry and Crandall,[9] who did not find an enzyme in the blood of the normal dog which

*Kindly supplied to us by Dr. G. S. Jamieson of the Bureau of Agricultural Chemistry and Engineering, U. S. Dept. of Agriculture.

splits olive oil. We believe the inability of the Cherry and Crandall test to demonstrate the presence of an olive oil-splitting enzyme in normal dog serum is due to the lack of sensitivity of this method. In the latter procedure the hydrolysate is buffered to produce an initial pH of 7, which is below the optimum range for lipase; this feature would reduce considerably the rate of hydrolysis and could account for the failure to obtain splitting of olive oil by normal dog serum.

After our comparative studies were under way, it was realized that tributyrin could be dispersed without the aid of bile, since this glyceride is slightly soluble in water. Experiments showed that tribuytrin, when properly homogenized in the aqueous buffered digestion mixture, is split by blood serum even more rapidly than when emulsified with bile. It was necessary to use bile in our comparative studies for the satisfactory emulsification of the other substrates, and to make a true comparison, tributyrin had to be emulsified in the same way. Certain advantages are to be gained by using an aqueous tributyrin suspension, however. The end point of the titration with the aqueous suspension is more easily discerned, and the difficulty of preparing and titrating a bile emulsion is obviated. If bile is not used as the emulsifying agent for tributyrin, an equal volume of water is substituted and the test is carried out in the same way. This change in technique will yield values for normal blood serum about twice as high as those we obtained with the use of bile as the dispersing agent. We plan to report later normal values for human serum by this method when an aqueous tributyrin suspension is used.

The relation of the amount of fatty acid liberated to the concentration of enzyme and to the time of hydrolysis in the proposed method is of interest. As twenty-four hours of hydrolysis are required when ethyl butyrate or triolein or olive oil is used as the substrate, and three hours of hydrolysis are required with benzyl butyrate as substrate, studies of time or concentration relationships would be somewhat unsatisfactory with these substrates. The tributyrin procedure, however, is well adapted to such studies, since it requires only one hour of hydrolysis. The studies we made were with tributyrin as substrate, the procedure being the same as outlined above.

The results of these studies are shown in Figs. 1 and 2. Exact linear relationship of the amount of fatty acid liberated to the time of hydrolysis, or to the concentration of enzyme, is not shown. Fairly good relationships are present in the first three hours of hydrolysis, however; also where small amounts of enzyme (0.5 c.c. and 1 c.c. of serum) are used.

The amount of substrate used in these studies is a marked excess. The 0.25 c.c. of tributyrin is equivalent to 26 c.c. of 0.1 N butyric acid; the same amount of triolein is equivalent to 7.4 c.c. of 0.1 N oleic acid. These amounts of fatty acid are 25 to 30 times that liberated in a hydrolysis by the enzymes of normal human serum under the conditions of the method described. The pH at the end of the hydrolysis in the experiment represented by Fig. 1 was 7.3; in the experiment of Fig. 2 the pH at the end of three hours was 7.4. Both of these pH values are within the optimum range. The deviations from straight line relationships in Figs. 1 and 2 must therefore be attributed to other conditions than the amount of substrate or the buffering of the reaction.

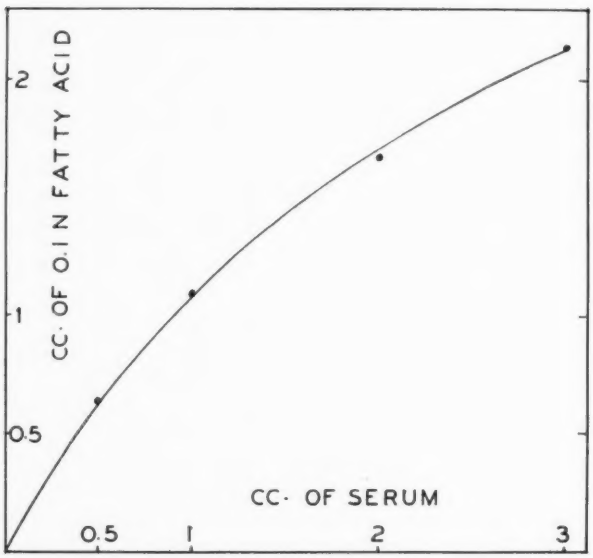

Fig. 1.—Relation of amount of fatty acid liberated to the concentration of enzyme under the conditions of the proposed method. Tributyrin was used as substrate, the time of hydrolysis being one hour. Human serum was the source of enzyme.

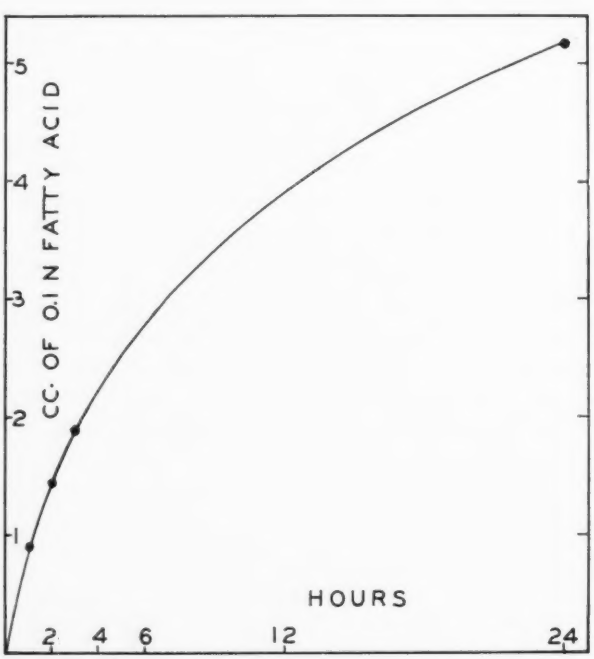

Fig. 2.—Relation of amount of fatty acid liberated to the time of hydrolysis under the conditions of the proposed method. Tributyrin was used as substrate, and cat serum was the source of enzyme.

SUMMARY

1. A study of the conditions entering into the determination of the lipidolytic enzymes of blood serum has been made. Attention was directed to the substrate, the emulsifying agent, the pH and suitable buffering, and the removal of end products.

2. In comparative studies of the five substrates, ethyl butyrate, tributyrin, benzyl butyrate, triolein, and olive oil, it was found that tributyrin is split most rapidly by the enzymes of blood serum.

3. A method for the estimation of the lipidolytic enzymes of blood serum is proposed. Using tributyrin as the substrate in this method, the amount of fatty acid liberated in one hour's hydrolysis by blood serum is comparable to the quantity obtained when a 24-hour hydrolysis of olive oil by the same serum is carried out.

4. This rapid test, involving a one-hour hydrolysis of tributyrin, may have clinical advantages. Further clinical work is necessary to appraise its possibilities.

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A SIMPLE METHOD FOR THE APPROXIMATE ANALYSIS OF CARBON DIOXIDE AND OXYGEN IN GAS SAMPLES*

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A SIMPLE apparatus which can be used by a relatively inexperienced technician to determine the carbon dioxide and oxygen content of samples of gas has been developed. The method of analysis is rapid and suitable for determinations where an accuracy of 0.3 per cent is adequate. This apparatus may be used for the determination of oxygen in samples of gas taken from cylinders containing mixtures of oxygen and nitrogen, and for the determination of carbon dioxide and oxygen in samples of gas collected from anesthetic apparatus, from oxygen tents, or from the thoracic cavity of patients with pneumothorax.

APPARATUS

The apparatus (Fig. 1) consists of a 10 c.c. burette calibrated in 0.05 c.c., on the top of which a stopcock (*A*) and a cup (*B*) have been annealed. It is advisable to have on hand at least two of these burettes in order that duplicate determinations can be made simultaneously.

The other pieces of equipment necessary to make the determinations consist of three 2,000 c.c. graduated cylinders, or hydrometer cylinders of similar size, in which the burettes may be completely immersed; three medicine droppers with fine capillary tips for filling the cups of the burettes and the rubber tubing connections; a ring stand equipped with a double spring burette clamp to support the burettes while introducing the solutions; a mouthpiece with a six-inch piece of rubber tubing attached to be used in filling the burettes with water; a 50 c.c. syringe and an appropriate needle for the collection of samples; and two short pieces (1.5 and 4 inches) of transparent rubber tubing and a screw clamp for use in the transfer of gas from the syringe to the burette or from a cylinder of oxygen and nitrogen directly to the burette.

CALIBRATION OF APPARATUS

In order to know the total amount of gas in the sample to be analyzed, it is necessary to determine the volume of the uncalibrated portion *C* (Fig. 1) of the burette between the zero mark and the stopcock *A*. This may be done by weighing the amount of water which the uncalibrated portion *C* of the burette will hold. To fill the burette, a short piece of rubber tubing and a mouthpiece, similar to that used with hematological pipettes, is attached to the capillary tip *D* below the stopcock *E*. The cup *B* is immersed in a beaker containing freshly

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boiled distilled water, which is drawn up by suction until the burette is completely filled. Care must be taken to prevent the presence of any air bubbles in the burette. Stopcocks *A* and *E* are closed, and the burette is placed in a burette clamp with the cup end up. Stopcock *A* is then opened, and, by careful control of stopcock *E*, just enough water is released so that the bore of stopcock *A* will contain air and the rest of the burette will be completely filled with water. The water is then discharged into a weighing bottle until the level of the liquid reaches the zero mark. The weight of the water and the temperature at which the weighing is made are recorded. An average of ten such weighings is corrected to the density of water at 25° C., and this value is used in the computation of the volume of the portion *C*.

The accuracy of the calibrations on the burette may be checked by the usual method of weighing the amount of water contained in each cubic centimeter.

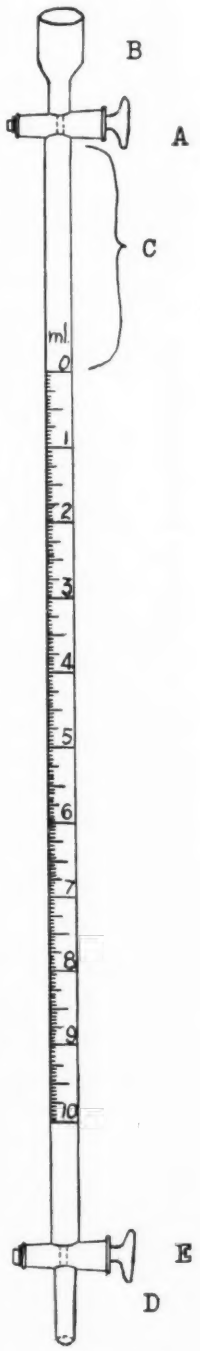

Fig. 1.—Diagram of burette used for the determination of carbon dioxide and oxygen in samples of gas. 1/3 actual size.

SOLUTIONS

A 15 per cent solution of either sodium or potassium hydroxide is used for the absorption of carbon dioxide. The potassium pyrogallate solution,[1] which is used for the absorption of oxygen, is prepared as follows: 450 Gm. of potassium hydroxide are dissolved in 300 c.c. of water, and, after complete solution has occurred, the liquid is thoroughly stirred, heated to boiling, and allowed to stand until it reaches room temperature. Then, 400 c.c. of this potassium hydroxide solution are added to 40 Gm. of pyrogallic acid in a 16 oz. widemouthed dark glass bottle. This is immediately stoppered, sealed with paraffin, and allowed to stand for a week before use. Since the solution keeps for two years or longer under these conditions, several bottles may be prepared at one time. When a bottle is opened for use, a rubber-stoppered washing bottle assembly is substituted for the original stopper. To prevent deterioration of the potassium pyrogallate between analyses, the ends of the glass tubing which project out through the stopper are protected from the air by medicine dropper bulbs. If care is thus taken to prevent undue exposure to air, this solution will keep in the washing bottle for at least nine months.

An oxygen absorbent,[2] which may be used immediately after it is made, consists of 16 Gm. of sodium hyposulfite, 13.3 Gm. of sodium hydroxide, 4 Gm. of anthraquinone β-sulfonic acid and 100 c.c. of water. When freshly prepared, it is just as satisfactory as the potassium pyrogallate. This alternate absorbent deteriorates rather rapidly, however,

even though it is protected from exposure to air by keeping it under mineral oil. Within one or two weeks a heavy precipitate develops during the analysis and thus prevents an accurate reading of the level of the solution.

COLLECTION OF GAS FOR ANALYSIS

The sample for analysis may be collected in a syringe and transferred to the burettes, or it may be taken directly into the burettes. The former procedure is used for the collection of pneumothorax air or of gas from an anesthetic apparatus; the latter, for the collection of air from an oxygen tent or from a tank containing a mixture of nitrogen and oxygen.

When a sample of pneumothorax air for duplicate analysis is to be collected, a sterile needle and a sterile 50 c.c. syringe must be used. The needle and the tip of the syringe are filled by drawing 1 or 2 c.c. of sterile water into the syringe, expelling any air bubbles that may have been admitted, and then emptying the syringe of water. The needle and the tip of the syringe are thus left filled with sterile water. The needle is inserted into the chest wall, and air is drawn into the syringe. After the needle is withdrawn, it is quickly removed from the syringe and a short piece of transparent rubber tubing, which has previously been clamped in the middle and completely filled with water, is immediately slipped onto the tip of the syringe. The transparency of the tubing permits one to be sure that no contaminating air bubbles have been admitted. After filling the burette with water in the manner described under the directions for its calibration, the unattached end of the rubber tubing, which should still be filled with water, is carefully slipped over the capillary tip *D*. Again care must be taken to exclude all air bubbles. The clamp on the rubber tubing is released and both stopcocks of the burette are opened while the cup end is held lower than the syringe. By pressing lightly on the plunger of the syringe, a sample of gas is transferred to the burette. When only about 4 c.c. of water remain in the burette, stopcock *A* and then stopcock *E* are closed. The tubing is clamped off and then removed from the burette. After again filling the free end of the tubing with water, a duplicate sample is transferred from the syringe to a second burette.

When gas from an anesthetic apparatus is to be collected, the needle and syringe need not be sterilized. The needle and the tip of the syringe are filled with water, and a sample of air is secured by inserting the needle in one of the rubber tubing connections. From this point, the procedure for transferring the gas to the burettes is the same as that described for pneumothorax air.

Air from an oxygen tent is collected directly into the burette. After the burette has been completely filled with water, the stopcock *E* is opened, and this end of the burette is inserted through the armhole of the tent to a point about midway between the face of the patient and the front wall of the tent. The burette is held at an angle of about 45 degrees, and the air sample is collected by permitting the water to run out of the lower stopcock *A* until about 4 c.c. remain. Stopcock *E* is closed as the burette is being withdrawn from the tent. In a similar manner, a duplicate sample is collected in a second burette.

In sampling gas from a tank containing a mixture of nitrogen and oxygen, a four-inch piece of transparent rubber tubing is connected to the outlet of the

tank. Room air is washed out of the valve of the tank by gently releasing gas for five or six seconds. The rubber tubing and the burette are completely filled with water, and the free end of the tubing is connected to the burette at tip *D*. If care is taken, this may be done without the introduction of room air. After opening stopcocks *A* and *E*, all but about 4 c.c. of water are forced out of the burette by a gentle stream of gas. The valve of the tank is shut off and the burette is disconnected after both stopcocks have been closed. In the same way a duplicate sample of gas is collected in a second burette.

PROCEDURE FOR ANALYSIS

The burettes containing the duplicate samples of gas are placed in the clamps with the cup ends uppermost. Approximately 2 c.c. of water are introduced into each cup with a medicine dropper. The upper stopcock of each burette is opened, and the lower stopcock *E* is then cautiously turned to permit enough water to run out to fill the capillary tip *D* with water. The lower stopcock must be closed while sufficient water remains in cup *B* to prevent the entrance of room air into the sample. After removing the excess water from the cup with a medicine dropper, the lower stopcock *E* of the burette is immersed in the water which almost fills the first graduated cylinder; the stopcock is opened; and the burette is lowered into the cylinder so that the entire burette up to the cup is covered with water. The two burettes are thus left in the water for two minutes in order that the gas may attain the same temperature as that of the water. In turn, each burette is lifted by the cup to prevent heating of the gas by the warmth of the hand, the exposed part is dried off with cheesecloth, and the height of the burette is adjusted so that the level of the water inside the burette is the same as that in the cylinder. The top (outer border) of the meniscus is read, because the bottom of the meniscus cannot be clearly distinguished later in the analysis after potassium pyrogallate is added. After the burette is read, the lower stopcock *E* is closed under water; the burette is withdrawn and again placed in the burette clamp. The second burette is handled in a similar manner. Approximately 2 c.c. of the potassium hydroxide solution are now admitted into each burette in the same way as the water was introduced. The burettes are removed from the clamps and inverted twice to assure contact of the gas with the potassium hydroxide. The burettes are then placed in the second graduated cylinder, and, after permitting all of the solution in each to drain to the lower end, readings are made as previously described. The burettes are removed, again inverted, returned to the graduated cylinder, and read a second time. If the two readings do not check within 0.02 c.c., the process is repeated. The difference between the initial and the final reading multiplied by 100 and divided by the sum of the initial reading and the volume of the uncalibrated portion *C* of the burette gives the per cent of carbon dioxide in the sample.

When check readings for carbon dioxide have been obtained, about 2 c.c. of potassium pyrogallate solution are blown into the cup of each burette from a bottle of this reagent that has been fitted with a washing bottle assembly. The pyrogallate is admitted to the burettes in the same manner as described for the

other solutions. The burettes are inverted twice, placed in the third graduated cylinder, and read as previously described. The process of inversion and reading is repeated until no change greater than 0.02 c.c. occurs in either burette on successive readings. The per cent of oxygen in the sample is calculated by taking the difference between the final reading for carbon dioxide and the final reading for oxygen, multiplying this difference by 100, and dividing by the sum of the initial reading (before the absorption of any gas has taken place) and the volume of the uncalibrated portion *C* of the burette.

When the analysis is completed, the burettes are thoroughly rinsed until the wash water no longer gives an alkaline reaction to litmus paper. Further analyses may be made immediately. From twenty to twenty-five minutes are required for making duplicate analyses and for washing the burettes in preparation for the next determination. If only one pair of duplicate analyses is to be made, just one cylinder needs to be used, since the loss of solutions into the water will not impair the accuracy of the next step in the determination. When the analyses for the day are completed, the stopcocks of the burettes are carefully greased and the cylinders are washed and filled with water, so that the air in the water will be lost by the time the next determinations are to be made.

ACCURACY OF THE METHOD

Table I gives results of analyses obtained on several different types of determinations and shows the variation to be expected by this method of analysis. It may be seen that the variation observed between duplicate analyses is not greater than 0.35 per cent. As a further check on the method, air from an open circuit chamber procedure for the determination of basal metabolism[3] has been analyzed both with the Carpenter-Haldane gas analysis apparatus[4] and with the procedure herein reported. Table II gives the results of 21 such analyses. The values found for carbon dioxide and for oxygen with the apparatus described check within 0.5 per cent of one another, and the mean values are within ±0.3 per cent of those obtained with the Carpenter-Haldane apparatus. It is evident from the results reported in Tables I and II that the method of analysis is suitable for determinations where an accuracy of 0.3 per cent is adequate.

TABLE I

ANALYSIS OF GAS FROM VARIOUS SOURCES BY THE SIMPLIFIED PROCEDURE DESCRIBED

SAMPLES FROM	CARBON DIOXIDE, PER CENT			OXYGEN, PER CENT		
	NO. 4 BURETTE	NO. 5 BURETTE	DIFFERENCE	NO. 4 BURETTE	NO. 5 BURETTE	DIFFERENCE
Oxygen Tent	1.59	1.21	0.32	41.21	41.48	0.27
Pneumothorax Air	0.0	0.0		20.96	21.05	0.09
Anesthetic Apparatus	4.31	4.26	0.05			
	2.88	2.80	0.08			
Rebreathing Experiment into Spirometer	3.68	3.94	0.26			
	4.78	4.96	0.18			
	3.36	3.30	0.06			
	2.93	2.67	0.26			
	0.72	0.75	0.03			
	1.94	1.90	0.04			
Tank of Oxygen and Nitrogen				12.54	12.23	0.31
				9.99	10.19	0.20
				12.11	12.45	0.34

TABLE II

COMPARISON OF SIMPLIFIED PROCEDURE WITH CARPENTER-HALDANE METHOD

ANALYSIS OF AIR COLLECTED FROM CHAMBER IN OPEN CIRCUIT PROCEDURE FOR THE DETERMINATION OF BASAL METABOLISM

CARBON DIOXIDE, PER CENT						OXYGEN, PER CENT					
SIMPLIFIED PROCEDURE				CARPENTER-HALDANE ANALYSIS (5)	DIFFERENCE BETWEEN COLUMNS 4 AND 5	SIMPLIFIED PROCEDURE				CARPENTER-HALDANE ANALYSIS (10)	DIFFERENCE BETWEEN COLUMNS 9 AND 10
NO. 4 BURETTE (1)	NO. 5 BURETTE (2)	DIFFERENCE (3)	MEAN (4)			NO. 4 BURETTE (6)	NO. 5 BURETTE (7)	DIFFERENCE (8)	MEAN (9)		
0.84	0.57	0.27	0.70	0.55	+0.15	20.21	20.66	0.45	20.44	20.36	+0.08
						20.92	20.97	0.05	20.94	20.97	−0.03
0.53	1.00	0.47	0.76	0.55	+0.21	20.53	20.42	0.11	20.48	20.36	+0.12
0.44	0.47	0.03	0.46	0.44	+0.02	20.41	20.92	0.51	20.66	20.46	+0.20
0.50	0.57	0.07	0.54	0.52	+0.02	20.56	20.23	0.33	20.40	20.37	+0.03
0.51	0.64	0.13	0.58	0.52	+0.06	20.06	20.46	0.40	20.26	20.37	−0.11
						20.84	21.05	0.21	20.94	20.97	−0.03
0.62	0.52	0.10	0.57	0.61	−0.04	20.62	20.29	0.33	20.46	20.32	+0.14
						21.26	21.26	0.00	21.26	20.97	+0.29
0.61	0.57	0.04	0.59	0.64	−0.05	20.48	20.73	0.25	20.60	20.28	+0.32
0.59	0.53	0.06	0.56	0.51	+0.05	20.19	20.48	0.29	20.34	20.37	−0.03
0.21	0.21	0.00	0.21	0.46	−0.25	20.33	20.57	0.14	20.45	20.46	−0.01
0.40	0.00	0.40	0.20	0.46	−0.26	20.20	20.60	0.40	20.40	20.46	−0.06
0.56	0.53	0.03	0.54	0.49	+0.05	20.68	20.17	0.51	20.42	20.41	+0.01
0.30	0.24	0.06	0.27	0.39	−0.12	20.22	20.46	0.24	20.34	20.51	−0.17
0.59	0.61	0.02	0.60	0.62	−0.02	20.08	20.22	0.14	20.15	20.28	−0.13
0.42	0.49	0.07	0.46	0.47	−0.01	20.17	20.65	0.48	20.41	20.44	−0.03
0.52	0.55	0.03	0.54	0.54	±0.00	20.42	20.46	0.04	20.44	20.34	+0.10
0.43	0.51	0.08	0.47	0.52	−0.05	20.34	20.49	0.15	20.42	20.37	+0.05
0.42	0.31	0.11	0.36	0.51	−0.15	20.38	20.53	0.15	20.46	20.34	+0.12
0.29	0.48	0.19	0.38	0.44	−0.06	20.29	20.36	0.07	20.32	20.47	−0.15

ADVANTAGES OF THE METHOD

Some of the advantages of the procedure described are listed as follows: (a) The apparatus can be made with ordinary laboratory equipment by any competent glassblower;* (b) the method is suitable for the analysis of gas containing carbon dioxide and oxygen in widely divergent concentrations; (c) regardless of the time elapsed since the last determination, the apparatus is ready for immediate use without the necessity of cleaning capillary tubing or of making a preliminary test for leaks as would be essential with many types of apparatus for gas analysis; (d) only from twenty to twenty-five minutes are required to make duplicate analyses; (e) after relatively little practice accurate results can be obtained by a technician who has had no previous experience in gas analysis.

SUMMARY

1. An apparatus and a method for the determination of carbon dioxide and oxygen, or both, in samples of gas are described.

2. The method is easy to use and duplicate analyses can be made in from twenty to twenty-five minutes.

3. The apparatus is ready for immediate use regardless of the time elapsed since the last determination.

4. The method is very satisfactory for determinations in which an accuracy of 0.3 per cent is sufficient.

5. The apparatus is valuable for making determinations of samples of gas taken from cylinders containing mixtures of oxygen and nitrogen, from anesthetic apparatus, from oxygen tents, or from the thoracic cavity of patients with pneumothorax.

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*If the expense involved is not a matter of consideration, an apparatus with the zero mark of the burette at stopcock *A* may be substituted for the one herein described. The only advantage of such a refinement would be that the total volume could be read directly; the accuracy of the apparatus would be in no way enhanced.

THE ESTIMATION OF THE ENZYMES, AMYLASE, PROTEINASE, AND LIPASE IN DUODENAL CONTENTS*

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SOON after the introduction of the duodenal tube as a successful method for obtaining duodenal contents, studies of the enzyme activities of this material were made. A variety of methods utilizing various chemical principles have been employed in such studies. In 1934 a committee appointed by the American Gastroenterological Association[1] conducted a study of the methods used to determine the enzyme activities of duodenal contents. A report of this committee stated: "It is obvious that this survey denoted the existence of unsolved technical difficulties in pancreatic enzyme analysis, otherwise there would be more unanimity of opinion favoring one system of tests. There is insufficient proof at the present time that we have a system of tests which is sufficiently accurate and simple to be routinely adopted for clinical purposes." The clinical usefulness of such enzyme studies has been recently reviewed.[2-4] The present investigation was undertaken to devise a system of enzyme methods that would employ optimal environmental conditions, would be applicable to use in a routine clinical biochemical laboratory, and could be readily evaluated by the clinician.

METHOD OF OBTAINING AND DILUTING DUODENAL CONTENTS

Duodenal contents may be readily obtained by means of a duodenal tube. The authors have made studies of the activities of fasting contents and contents obtained after stimulation of pancreatic secretion with olive oil introduced into the duodenum.[5, 6] Other investigators have utilized fasting contents[7] as well as contents obtained after using a variety of stimulants including magnesium sulfate, dilute hydrochloric acid, peptone solution, corn oil, olive oil, cream, beef bouillon, secretin,[8-12] and mecholyl.[11]

The sample to be analyzed is refrigerated between the time of obtaining from the patient and the time the analyses are to be made. Repeated studies have indicated that there is no significant alteration in enzyme activity of duodenal contents during a period of twenty-four hours, provided the sample is not strongly acid due to contamination with gastric juice. In such cases the results are quite unreliable even though the analyses are made immediately. One c.c. of the sample is made up to a volume of 50 c.c. by adding 10 c.c. of 0.2 molar phosphate buffer (pH 7.2) and distilled water. The 1:50 diluted sample is added to 9.5 c.c. of 0.2 molar phosphate buffer (pH 7.2) to give a

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1:1,000 dilution. This dilution is used for the determination of amylase. It is important to make the dilution with the buffer rather than with distilled water, since marked destruction of amylase may occur when samples are diluted with distilled water.

REAGENTS

1. *General Reagent*

A. 0.2 molar phosphate buffer, pH 7.2, prepared by dissolving 7.62 Gm. of anhydrous potassium dihydrogen phosphate and 20.45 Gm. of anhydrous disodium hydrogen phosphate in water to give a total volume of 1 liter.

2. *Reagents for Amylase*

A. One per cent soluble starch solution, prepared by suspending 1 Gm. of soluble starch (Merck, according to Lintner) in about 5 c.c. of cold water and then pouring about 90 c.c. of boiling water into the suspension. This solution is boiled for exactly one minute and then made up to a volume of 100 c.c. This solution should be refrigerated and should be prepared fresh approximately every two weeks. Even after standing in the refrigerator for a period of about three days the starch solution acquires a turbidity. If the flask containing the solution is heated for a minute in a boiling water bath, however, the turbidity will disappear and the digestibility of the solution will be the same as a freshly prepared solution.

B. Standard glucose solution which is a saturated solution of picric acid containing 1 mg. of glucose in each 3 c.c. of solution.

C. 0.5 molar sodium chloride solution.

D. Saturated solution of sodium carbonate.

3. *Reagents for Proteinase*

A. Five per cent casein solution, prepared by suspending 5 Gm. of casein (Merck, according to Hammarsten) in about 50 c.c. of water and then adding 40 c.c. of 0.1 normal NaOH and sufficient water to make a total of 100 c.c. This solution should be refrigerated and should be prepared fresh about every two weeks.

B. Twenty per cent solution of trichloro-acetic acid.

C. Nessler's solution prepared by method of Folin.[13]

D. Digestion mixture for micro-Kjeldahl determination consisting of 50 per cent sulfuric acid containing 0.25 per cent copper sulfate.

E. 0.3 per cent gum ghatti solution, used for micro-Kjeldahl determination.

F. Nitrogen standard containing 0.283 Gm. of ammonium sulfate per liter (0.3 mg. of nitrogen in 5 c.c.).

G. Standard tyrosine solution containing 0.15 mg. of tyrosine in 3 c.c. Method of preparation described by Anson and Mirsky.[15]

H. Phenol reagent of Folin and Ciocalteau.[14]

I. 0.5 N sodium hydroxide solution.

(Solutions C, D, E, and F are needed only if the micro-Kjeldahl determination of nonprotein nitrogen is employed in the proteinase method.)

4. *Reagents for Lipase*

A. One per cent bile salt solution, prepared by dissolving 1 Gm. of bile salts (Wilson Laboratories, Chicago) in 100 c.c. of water. This solution should be refrigerated, and should be prepared fresh approximately every two weeks.

B. 0.1 normal sodium hydroxide in alcohol, prepared by adding sufficient saturated solution of sodium hydroxide to 95 per cent alcohol to give a 0.1 normal solution. The base is standardized against 0.1 normal acid in the conventional manner.

C. Tributyrin, obtained from Eastman Kodak Co., Rochester, New York.

METHOD FOR DETERMINATION OF AMYLASE

The principle of the method for the determination of amylase is based on the measurement of the amount of reducing sugar formed when a sample of diluted duodenal contents is incubated with a solution of soluble starch. Under proper environmental conditions, the amount of reducing sugar formed is proportional to the amount of amylase present so that this figure furnishes an index of the enzyme concentration.

Add 6 c.c. of 1 per cent soluble starch to a test tube along with 1 c.c. of 0.5 molar sodium chloride and 2 c.c. of phosphate buffer (pH 7.2 to 0.2 M). This mixture is warmed to 38° C. in a constant temperature water bath or a constant temperature oven and then 1 c.c. of the 1:1,000 diluted duodenal contents is added. The tube is thoroughly mixed and replaced in the water bath for exactly thirty minutes. At the end of this time a small amount of dry solid picric acid (about 0.5 Gm.) is added and the contents of the tube saturated with picric acid by continued mixing. This effectively stops enzyme action and provides a saturated picric acid solution for the determination of reducing sugar. Three c.c. of the supernatant solution are pipetted into a Myers-Bailey sugar tube, and 1 c.c. of saturated sodium carbonate solution is added. A standard is prepared by taking 3 c.c. of the glucose standard in picric acid and adding to it 1 c.c. of the saturated sodium carbonate solution. The tubes are placed in a boiling water bath for exactly twenty minutes to effect color development. The standard is diluted to 10 c.c. and the unknown to either 10, 15, or 20 c.c., following which the solutions are compared in a colorimeter. The equivalent of glucose which is produced in the digestion is calculated by the equation $S/R \times D/10 \times 1.0 \times 10/3 =$ mg. of glucose equivalent. A blank which represents the reducing action of the starch must be determined and subtracted from the above value. This will be quite constant for a given batch of starch solution on any particular day and can be readily determined by carrying through the entire procedure described above for the digestion mixture with the exception that the diluted duodenal contents are not added to the reaction mixture until after the precipitation with picric acid. Since all of the reducing value of the blank is contributed by the starch solution, one blank is sufficient for a series of determinations.

METHOD FOR THE DETERMINATION OF PROTEINASE

Proteinase is determined by incubating diluted duodenal contents with a solution of casein and then determining the amount of protein digestion products formed. The index of proteinase concentration is taken as the amount of nonprotein nitrogen formed from the casein or as the amount of material formed which will react with the phenol reagent.

Three c.c. of casein solution are added to a test tube along with 3 c.c. of water and 3 c.c. of phosphate buffer (pH 7.2 to 0.2 M). The test tube is placed in the constant temperature water bath at 38° and allowed to reach equilibrium temperature. To the tube is now added 1 c.c. of 1:50 diluted duodenal contents and the mixture thoroughly stirred. The tube is replaced in the bath and digestion is allowed to proceed for exactly thirty minutes, at the end of which time the tube is removed and 2 c.c. of 20 per cent trichloro-acetic acid are added. This serves to stop the enzyme action as well as to precipitate the undigested casein. The solution is filtered and either a 1 c.c. or a 2 c.c. aliquot is used for a nitrogen determination according to the micro-Kjeldahl procedure described later, or a 1, 2, or 3 c.c. aliquot is used for the colorimetric phenol reaction described later. A choice of either of these methods is offered depending on the facilities of the laboratory, since it has been found as a result of several hundred analyses that the two methods give the same results. It is the authors' belief that if the laboratory routinely carries out determinations of nonprotein nitrogen on blood the micro-Kjeldahl nitrogen estimation might be the method of choice whereas otherwise the colorimetric phenol determination would be more convenient.

For the determination of nonprotein nitrogen the following procedure is employed: An aliquot of filtrate is placed in a large Pyrex test tube calibrated at 35 c.c. and 50 c.c. One cubic centimeter of sulfuric acid digestion mixture is added along with a glass bead, and the mixture is digested over a flame or on a hot plate until perfectly colorless. Two or three drops of superoxol are added at intervals to facilitate the digestion. The digestion material is cooled, some water and 1 c.c. of gum ghatti solution are added, and the solution made up to 35 c.c. A standard is prepared in the following manner: Five c.c. of standard nitrogen solution are measured into a similar tube, 1 c.c. of the digestion mixture added along with 1 c.c. of gum ghatti and diluted to 35 c.c. Fifteen c.c. of dilute Nessler's solution (Folin) are added to both the unknown and the standard, and the colors are compared in a colorimeter. The calculations are based on the formula: $S/R \times 12/A \times 0.3 =$ mg. of nitrogen produced in the digestion. A is the volume of the aliquot used for the nitrogen determination.

Casein solutions prepared according to the above method contain small amounts of nonprotein nitrogenous substances. Correction for this is made by running a blank digestion in exactly the same manner as described above with the exception that the diluted duodenal contents are added after the precipitation with trichloro-acetic acid. This is filtered, and the nitrogen is determined in the filtrate according to the method described. One blank is sufficient for a series of determinations, and this blank value is subtracted from the total nonprotein nitrogen.

If the proteinase activity is to be determined by the colorimetric phenol reaction, the following procedure is employed: Three c.c. of the trichloro-acetic acid filtrate are measured into a test tube, to which are added 6 c.c. of 0.5 N NaOH and 2 c.c. of diluted phenol reagent diluted 1 to 3 with distilled water just before using. A blue color develops spontaneously; this should be compared after ten minutes with a standard solution of tyrosine. The stand-

ard is prepared by treating 3 c.c. of tyrosine standard with sodium hydroxide and phenol reagent in the same manner as the filtrate. Where the amount of proteinase activity is relatively large, the color of the unknown will be considerably darker than that of the standard and will thus result in a certain error due to the disproportionality of the colors. In this case the use of a 1 c.c. aliquot of filtrate is necessary. In order to make the final volume the same, as well as to secure the proper reaction, 2 c.c. of a mixture of phosphate buffer, trichloro-acetic acid, and water in the proportion in which they occur in the filtrate are added to the tube containing the 1 c.c. aliquot. This is then made alkaline, and the color is developed in the usual manner. If this is done, the proportionality of enzyme concentration to the intensity of color developed will hold. Of course, the use of a smaller amount of filtrate must be taken into account in the calculations. The calculation of the amount of proteinase activity may be made by the following formula: $S/R \times 12/A \times 0.15 =$ mg. of tyrosine equivalent produced in the digestion.

A blank determination is necessary, since the casein solution will yield a filtrate after precipitation that contains a small quantity of substances which react with the phenol reagent. The blank is prepared according to the method described above, and the filtrate is then subjected to the same treatment which is given to the digest filtrate. The value of the blank is subtracted from the value obtained in the digestion mixture.

METHOD FOR THE DETERMINATION OF LIPASE

Lipase is determined by incubating duodenal contents with tributyrin and then titrating the amount of acid liberated. The index of lipase concentration is calculated from the amount of acid released.

Into a 200 mm. test tube are measured 4 c.c. of phosphate buffer, 1 c.c. of 1 per cent solution of bile salts, and 5 c.c. of 1:50 diluted duodenal contents. The tube is warmed for three or four minutes in the constant temperature bath at 38° C. One c.c. of tributyrin is then added, and the contents of the tube are thoroughly agitated. The agitation of the tube at this point is important, since it is responsible for the formation of a semiemulsion which is required for the reaction. The tube is replaced in the constant temperature bath and enzyme action is allowed to proceed for one hour, during which time the temperature of the bath is maintained at 38° C. At the end of this time the tube is removed, and 5 c.c. of alcohol and a few drops of phenolphthalein are added. The contents are then immediately titrated with 0.1 N NaOH in 95 per cent alcohol to the first color change of the phenolphthalein.

Since phosphate buffer is present, there will be a small blank titration even though no lipase digestion has occurred. This amounts to approximately 2.75 to 3.00 c.c. of 0.1 N alcoholic NaOH. When this blank is subtracted from the titration, the resulting value gives a measure of the lipase activity. Since the blank is entirely due to the phosphate buffer and tributyrin, one blank will suffice for a series of determinations.

With the environment described for the lipase estimation, the relation between the amount of acid liberated and the amount of enzyme present is not linear. Therefore, it is necessary either to prepare a graph and read the values from the graph or to use a table and obtain the values from the table.

This, although somewhat troublesome, does not impair the accuracy of the final results obtained by this method.

VARIATIONS OF ENZYME ACTIVITY WITH VARYING ENZYME CONCENTRATION

In both the amylase and proteinase method there is a linear relation between the index of enzyme activity and the enzyme concentration over the range of values encountered in normal and abnormal samples of human duodenal contents. This relation is shown in Figs. 1 and 2. Therefore, in the case of amylase determinations, the amount of reducing sugar produced by the digestion can be used as an index of enzyme concentration. Similarly in the proteinase determination the amount of nonprotein nitrogen liberated from the casein or the amount of tyrosine-like material liberated can be used as an index of enzyme concentration. In the lipase method, however, there is not a direct linear relation between the amount of enzyme present and the amount of acid liberated. The curve for the variation of acid liberated with variations in enzyme concentration is shown in Fig. 3. Therefore, in order to secure an accurate index of lipase concentration, it becomes necessary to use the curve or to employ a table such as that indicated by Table I.

STUDIES OF FACTORS INFLUENCING ENZYME ACTIVITY

Many of the steps in carrying out determinations of enzymes are quite arbitrary, since the methods for enzyme measurement are based on determination of enzyme activity. By variation of each of the arbitrary steps in the procedure it is possible to obtain a method which estimates the enzyme under optimal environmental conditions. Fig. 4 shows the effect of variations in pH on the activity of amylase in duodenal contents. It will be seen that when the digestion mixture is buffered with phosphate buffer of pH 7.2, there is an optimal conversion of starch to reducing sugar. Therefore, this pH was employed in the amylase determination. Since optimal digestion by proteinase and lipase also occurs at this reaction, the same buffer is used in the procedure for the estimation of proteinase and lipase. A complete description of the experimental studies that have been carried out in the development of the methods here described has been reported elsewhere.[16] It should be stated that not only is the pH optimal, but also the substrate concentration, buffer concentration, salt concentration, and the amount of duodenal contents employed are optimal for the methods described.

ENZYME ACTIVITIES OF DUODENAL CONTENTS OF NORMAL SUBJECTS

When fractional samples of duodenal contents are obtained after stimulation of pancreatic secretion with olive oil, there is a considerable variation in the enzyme activity of various fractional samples. For this reason the maximum enzyme concentration of the fractional samples has been selected as a more constant criterion of potential enzyme activity.[6] The maximum amylase, proteinase, and lipase values obtained in 17 studies made on 11 normal subjects are shown in the first three columns of Table II. The average maximum enzyme activities for these normal subjects are amylase, 10.99 mg. of glucose equivalent; proteinase, 2.24 mg. of tyrosine equivalent; and lipase, 13.92 c.c. of 0.1 N acid liberated (uncorrected).

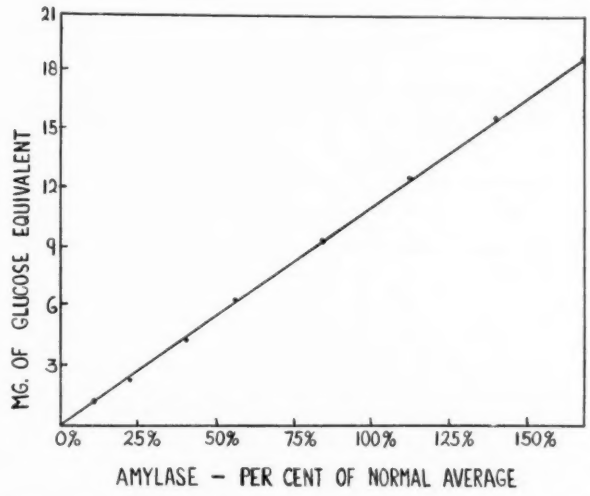

Fig. 1.—Relation of amylase activity to amylase concentration. The variations in enzyme concentration were attained by using different amounts of the same sample of duodenal contents. For convenience the various concentrations are expressed as a per cent of the normal average. Amylase activity is expressed as mg. of glucose equivalent formed in the digestion mixture.

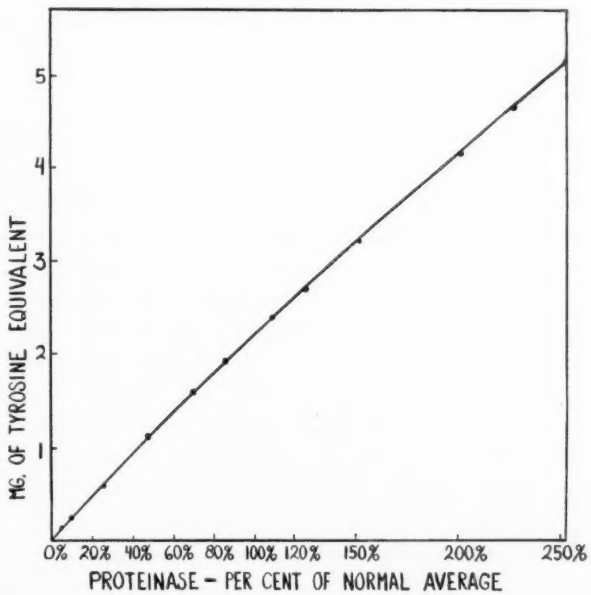

Fig. 2.—Relation of proteinase activity to proteinase concentration. The variations in enzyme concentration were attained by using different amounts of the same sample of duodenal contents. For convenience the various concentrations are expressed as a per cent of the normal average. Proteinase activity is expressed as mg. of tyrosine equivalent formed in the digestion mixture.

The authors have proposed a simplified method of expressing enzyme values.[4-6] According to this system any individual finding is expressed as a percentage of the normal average value. On this basis the values for the normal subjects are shown in the last three columns of Table II. The conversion of amylase or proteinase figures to this system is quite simple and merely involves multiplying by a factor. The factor for the conversion of mg. of glucose equivalent to amylase as a per cent of the normal average is 9.1. For proteinase determined from the tyrosine reaction one needs to multiply the mg. of tyrosine equivalent by the factor 44.4. If the amount of nonprotein nitrogen formed by the proteinase is measured (the average amount of nonprotein nitrogen obtained in the normal subjects is 4.70 mg.), the factor is 21.3. The values for lipase as a per cent of the normal average can be approximated from the data given in Table I or from Fig. 3.

Using this method of comparison the range of variation of amylase is from 55 per cent to 165 per cent of the average normal, whereas proteinase varies from 45 per cent to 148 per cent while lipase varies from 46 per cent to 147 per cent. In the subjects in whom more than one study was made there was considerable variation in the maximum enzyme concentration, but this variation was less than that observed in the different subjects.

This system of expressing results of enzyme studies as a per cent of the normal average can also very readily be applied to results obtained in patients as has been done by the authors.[6] It thus greatly facilitates the clinical interpretation of such data. This method is quite logical, since enzyme measurements determine relative rather than absolute concentrations, the relative concentration being derived from the activity of the enzyme.

DISCUSSION

It should be pointed out that the principles of the methods that are described above are not new. In certain respects the amylase determination makes use of the same principles employed by Myers and Killian[17] in the determination of blood amylase or diastase. Picric acid reduction is particularly well adapted for the measurement of the substances resulting from starch digestion, since maltose, which is the principal product of amylolysis, exerts approximately twice the reducing effect on picric acid that it does on most copper reagents.[18] The use of sodium chloride in the digestion mixture enhances the activity of the amylase and removes the possibility that with some samples the chloride content might influence the measured amylase activity. The use of a pH of 7.2 gives a much greater activity than that obtained at other reactions, such as the pH of 8.4 employed by McClure, Wetmore, and Reynolds.[19] This is clearly demonstrated in Fig. 4.

The proteinase method is in certain respects patterned after the proteinase methods of McClure, Wetmore, and Reynolds,[19] and Anson and Mirsky.[15] The latter investigators employed hemoglobin as a substrate and maintained that this is the only reproducible substrate for proteinase estimations. The reproducibility of casein as employed in the proteinase method has been carefully investigated by means of an experiment in which several different batch preparations of Merck's Casein, according to Hammarsten, were obtained and were used as substrates with the same sample of duodenal contents as a source of enzyme.

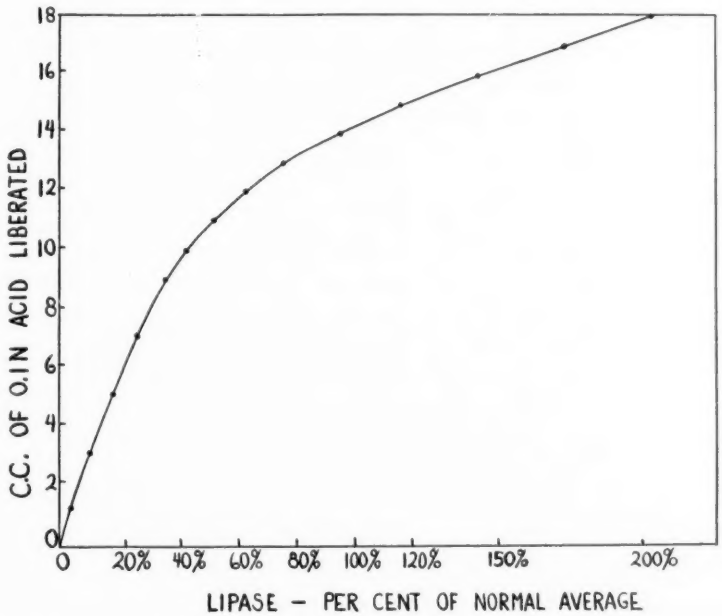

Fig. 3.—Relation of lipase activity to lipase concentration. The variations in enzyme concentration were attained by using different amounts of the same sample of duodenal contents. For convenience the various concentrations are expressed as a per cent of the normal average. Lipase activity is expressed as the c.c. of 0.1N acid liberated in the digestion mixture.

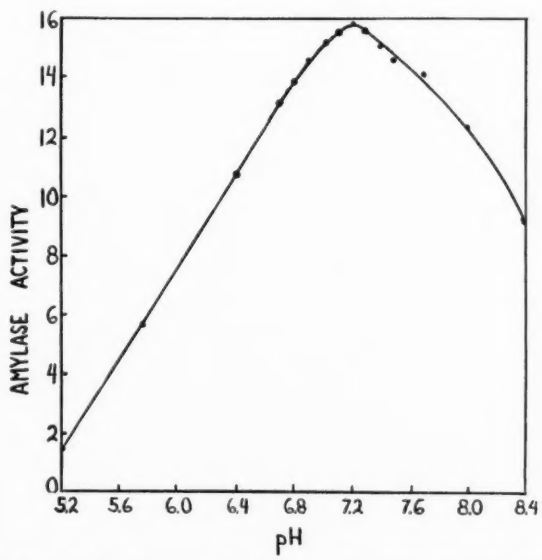

Fig. 4.—Effect of pH on amylase activity. Variations in pH were accomplished by varying the ratio of the acidic and basic salt in the phosphate buffer. All other factors were maintained constant.

The values obtained with the different samples of casein were identical. A sample of casein prepared in the laboratory by a comparable technique also gave a satisfactory result. Experimental data also indicated that a casein solution prepared by the described technique could be used for as long as three weeks without any change in digestibility, although during this time there was some increase in the value of the blank.

TABLE I

RELATION BETWEEN ACID LIBERATED FROM TRIBUTYRIN AND LIPASE ACTIVITY

TITRATION AMOUNT OF 0.1N ACID LIBERATED	AVERAGE NORMAL LIPASE PER CENT
1 c.c.	2.6
3 c.c.	9.0
5 c.c.	17.5
7 c.c.	26
9 c.c.	35
10 c.c.	43
11 c.c.	53
12 c.c.	64
13 c.c.	77
14 c.c.	97
15 c.c.	118
16 c.c.	145
17 c.c.	175
18 c.c.	219
19 c.c.	272

TABLE II

MAXIMUM ENZYME ACTIVITIES OF NORMAL SUBJECTS

SUBJECT	AMYLASE MG. OF GLUCOSE EQUIVALENT	PROTEINASE MG. OF TYROSINE EQUIVALENT	LIPASE C.C. OF 0.1N ACID LIBERATED (UNCORRECTED)	AMYLASE PER CENT OF AVERAGE	PROTEINASE PER CENT OF AVERAGE	LIPASE PER CENT OF AVERAGE
A. H. F.	11.2	1.90	12.50	102	85	70
A. H. F.	14.4	2.29	15.25	131	102	124
A. H. F.	11.5	2.24	14.05	105	100	97
A. H. F.	13.5	2.91	14.20	123	130	101
J. M. C.	7.05	1.68	11.30	64	75	55
J. M. C.	8.36	2.17	14.25	76	97	102
J. M. C.	6.65	2.01	13.25	60	90	83
N. H.	11.9	2.35	11.85	108	105	61
N. H.	9.6	1.70	16.10	87	76	147
W. E. B.	6.03	1.45	12.85	55	65	74
R. K.	18.2	3.12	15.85	165	139	140
D. H. F.	8.82	1.01	10.10	80	45	46
E. W. M.	8.25	2.47	15.95	75	110	145
L. J.	12.4	3.32	15.70	112	148	137
E. J.	17.5	3.30	15.40	158	146	130
H. E.	10.6	2.04	14.40	96	91	104
I. A.	10.8	2.29	13.70	98	102	90
Average	10.99	2.25	13.92	100	100	100

The lipase method employs the same principle that has been made use of in a large number of methods of lipase estimation. In most of these methods the amount of acid liberated by the hydrolysis of a substrate catalyzed by lipase has been measured. Chace and Myers[20] used ethyl butyrate as a substrate; McClure, Wetmore, and Reynolds[19] employed cotton seed oil; Wads-

worth and Aaron[21] utilized triacetin; and Cherry and Crandall[22] used olive oil emulsion. There have been several objections raised to the use of tributyrin[22] as a substrate for lipase estimations, since it has been maintained that the digestion of tributyrin is accomplished by an esterase and not by the lipase involved in the splitting of higher fats. In a series of approximately 500 determinations in various normal and abnormal samples of duodenal contents by the method described above, parallel determinations of lipase by a method quite similar to that described were made in which the substrate was an olive oil emulsion. In every one of the samples studied the amounts of acid liberated from tributyrin and from olive oil emulsion were practically identical. In no instance would the clinical interpretation of the finding be any different whether olive oil or tributyrin was used. Since tributyrin is somewhat more convenient to obtain and handle, the method using tributyrin is preferred by the authors to the method using olive oil emulsion. The addition of bile salts to the digestion mixture is designed to eliminate any effect the amount of bile in a sample of duodenal contents might have on lipase activity.

Intestinal contents obtained by means of a Miller-Abbott tube from an isolated segment of intestine[23] have been studied in an endeavor to ascertain whether intestinal secretions when uncontaminated with pancreatic juice possess any enzymes that will give appreciable enzyme activity under the environmental conditions described in the above methods. Intestinal secretions obtained from two normal human subjects did not show any appreciable enzyme activity by the methods described. This would indicate that alteration in the enzyme concentrations of duodenal contents are entirely due to variations in the enzyme concentration of the pancreatic juice or variations in the amount of pancreatic juice in relation to the total volume of duodenal contents.

There is a considerable range of variation in the enzyme activity of duodenal contents of normal human subjects. This makes the definition of the pathologic values quite difficult. By a combination of factors we believe that we are able to define more accurately the normal variations and thus obtain an earlier recognition of pathologic values. These factors include: (1) the use of sensitive accurate enzyme methods; (2) the elimination of certain variability of pancreatic secretory activity by using the maximum enzyme activity of fractional samples; and (3) the expression of the values by a convenient system which permits evaluation on the basis of the normal average. This has been demonstrated by data on a group of fifteen miscellaneous cases, given in Table V of the paper,[6] presenting observations obtained with these methods.

SUMMARY

Methods are described for the estimation of the enzymes of the duodenal contents, amylase, proteinase, and lipase, in which an optimum pH and optimum concentrations of substrate, buffer, and salt are employed. The values for the enzyme activities are recorded in terms of the normal taken as 100, and thus permit ready interpretation by the clinician.

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MEDICAL ILLUSTRATION

NEW IMPROVEMENTS IN MOULAGE PROSTHESIS*

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THE word *prosthesis* means an artificial organ, such as an eye, nose, ear, hand, leg, or denture that is used to replace an absent part in the body.

In reprinting one of my articles entitled *Moulage Prosthesis,* the editor of the publication changed the title to *Facial Prosthesis.* This was undoubtedly done for clarification because the word *moulage* is not fully understood. Any author working on the subject of molding and casting is plagued with the necessity for the continued use of these two words. Sometimes he varies them with positive and negative. The word *moulage* has come to be known by a select few in the medical sciences as a wax cast. There are, however, more durable and lifelike mediums than wax. Wax is rapidly becoming a substance for admixing with other ingredients to produce an intermediate stage, pattern, or model in the molding and casting art. Rubber and synthetic plastics, such as *catalin,* are being used for the finished product. To prevent the necessity for repeatedly referring to the two distinct steps, molding and casting, as a unit, the word *moulage* is used to express both. It is of French origin (mouler), meaning to cast or to mold. It should be understood, however, that molding and casting are processes for reproducing an object in form, texture, and often in color, identical in appearance with the original from which the copy or reproduction is made. The mold is the negative; the cast is the positive. The words *pattern* and *model* are used to express the original from which the mold is made. They are used for clarification and amplification, and to prevent repetition and confusion. Since it is possible to make molded and cast prostheses of hands, and casts to cover up scar depressions visible through thin hose on the legs of women, the subject as a whole will be referred to as *moulage prosthesis.* The word *moulage* differentiates the molded and cast prosthesis from the prosthetic appliance which is purely mechanical, such as constructed artificial legs or hands crudely carved from wood and worn with gloves.

Rubber Latex.—The use of rubber latex finally has been established as one of the best materials for the reproduction of artificial parts to be worn on the human body. Synthetic rubber (neoprene) latex is also being employed for this purpose at present. The first mention of rubber latex was made by Clarke[1] and elaborated upon by Bulbulian.[2, 3] In 1936 Mr. Gilbert Blumberg was sent from the Department of Art of the School of Medicine, University of Maryland, to the Mayo Clinic to do moulage work in agar, wax, rubber, and similar

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materials. Bulbulian evidently used the rubber materials employed there by Blumberg. Further articles by Clarke[4, 5] were written to simplify the technique and to establish a knowledge of the wearing qualities of latex prostheses. Up to this time vulcanite, a hard opaque milled and vulcanized rubber material, was used extensively by dentists for the making of false gums, for holding porcelain teeth, for artificial palates, and similar devices. Kazanjian, Rowe, and Young[6] wrote an interesting article on vulcanite for use in preparing prostheses of the nose and areas around the eyes. Flesh is translucent. This fact can be established by holding the fingers over a burning incandescent electric bulb or flashlight head in a darkened room. Unfortunately, vulcanite is rigid and opaque. It will not move with the face as it assumes various expressions. The prosthesis for the face, if properly made of rubber latex, is soft, flexible, and more fleshlike in appearance. It lends itself more readily to the application of cosmetics and for these reasons is more suitable than vulcanite. Rubber latex can also be made suitably stiff for prosthetic arms, hands, and fingers.

Rubber latex, however, has its own shortcomings and difficulties. Unless properly prepared it darkens on exposure to sunlight. Fugitive or nonpermanent colors used in simulating flesh tones will bleach from the set prosthesis. Shrinkage will occur. Seam lines may appear on the surface of a cast where two or more pieces constitute the mold. All of these disadvantages can be overcome. The methods of doing so will be described. No amount of description or the release of so-called trade secrets, however, will take the place of knowledge gained from the actual working with the necessary materials and from carefully planned and executed workmanship. The information herewith given is substantiated by hundreds of tests and experiments. This approach to the subject is presented because few processes are foolproof. For example, plaster of Paris may be added to water to obtain a homogeneous mixture, but it is more difficult to prepare a smooth mixture if water is added to a quantity of plaster of Paris. Nevertheless, the latter process has been attempted by the novice on numerous occasions. An elementary knowledge of moulage practices is absolutely essential. For this reason *Molding and Casting*[1] is strongly recommended as a reference.

The making of agar molds has been described fully[7] and will not be discussed here. The steps required for producing the prostheses from plaster molds also have been explained.[4] Prostheses or positives of many kinds, however, can be produced from many types of molds, such as those made of rubber, wax, glue, metal, and synthetic plastics.

Because of the demands of World War II the supply of agar has become somewhat limited. Other materials are being substituted for molding and casting with varying degrees of success. An extractive of the seaweed *Chondrus crispus,* commonly known as Irish moss or kelp, is being used in combination with agar in different proportions for the best results. Considerable research should be done on Irish moss to perfect formulas more suitable for moulage purposes.

Another derivative of kelp that is proving more successful than Irish moss is algin or algic acid. It is a protein of marine algae obtained as a by-product

in the preparation of iodine from kelp. Those interested in pursuing this subject should consult American patent No. 2,249,694, "Material for Taking Impressions for Dental and Other Purposes," issued July 15, 1941.

Fig. 1.—A plaster cast is made of the patient.

RUBBER MOLDS AND CASTS

Gum Rubber.—Rubber alone is seldom used for the making of rubber products. It invariably is compounded with other ingredients and these materials greatly influence the properties, quality, and cost of the finished article. It is necessary to mix them with the dried rubber gum by milling, or to the latex or latex paste by agitation. Since this text does not discuss dried rubber, only latex will be considered. The ingredients for mixing with latex are divided into two forms, powders and liquids. Often the powders are converted into liquid or paste mixes for convenience of incorporation; otherwise, the powders may coagulate the latex when stirred into it or the latex will coagulate when stirred into the powder. Of course, this greatly depends on the concentration

of the latex or its dry rubber content, which is spoken of in relation to the *total solid* content of the latex and is an important consideration at all times.

Rubber latex may be procured in two forms,* the unvulcanized latex and the vulcanized latex, often spoken of as prevulcanized latex. The latter form is the simplest to use, but the methods of using both are herewith given.

Fig. 2.—A wax nose is modeled onto the plaster cast or molded and cast from a donor and put in place on the plaster cast.

Unvulcanized Latex.—The unvulcanized or common rubber latex generally requires, in addition to fillers, a vulcanizing ingredient (sulfur), an agent to speed vulcanization (accelerator), a thickening agent (zinc oxide), and a preserving agent (antioxidant). A simple formula for this purpose is:

Rubber, such as latex concentrate	100	parts
Sulfur, flowers of	2	parts

*At present the use of rubber is greatly restricted because of the war. The amount required for prosthetic work is so small and is employed for such an excellent purpose, however, that an application may be made through the Rubber Reserve Board for the small quantities necessary for this work.

Accelerator (Captax)	0.5	parts
Zinc oxide	3	parts
Antioxidant	1	part
Color	q.s.	

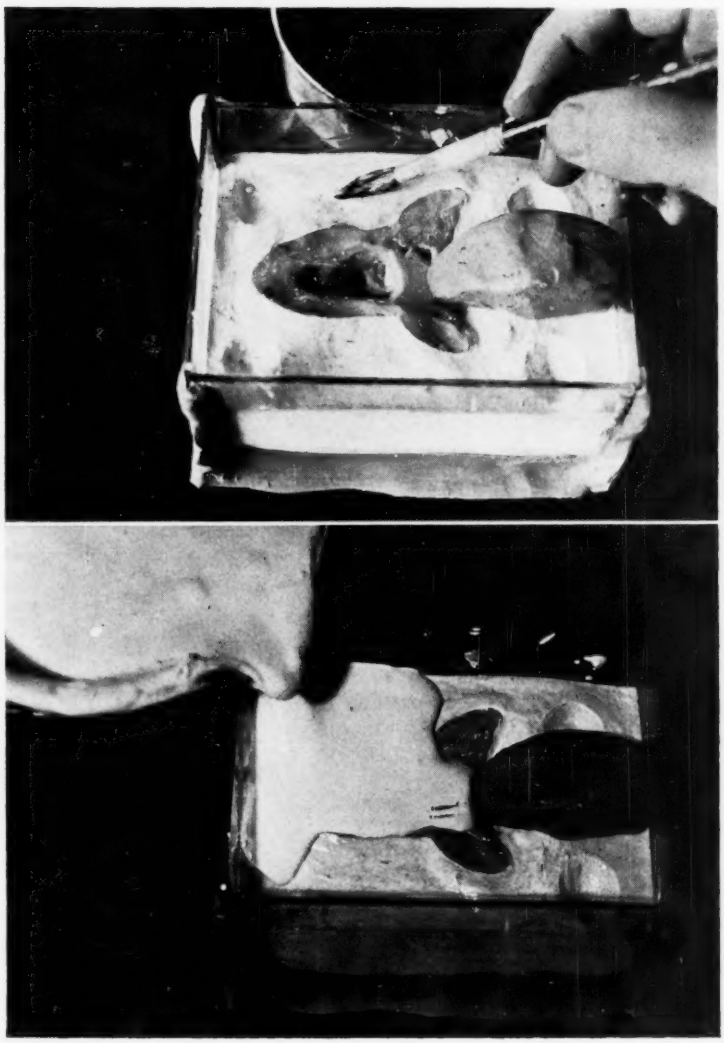

Fig. 3.—*A*. The wax nose is removed from the plaster cast and painted with a plaster-water mixture on one side only. It is then imbedded into a plaster-water mixture in a box made of glass. Registration notches are cut into the set plaster and a separating medium is applied. A piece of plasteline is put in place to form a pouring funnel in the second half of the plaster mold.

B. The second half of the mold is made.

To this mixture can be added fillers, such as clays, cellucotton, and other ingredients, to increase its bulk and to make it pastelike so that it will remain on a vertical surface to suit specific purposes. A clay filler usually serves best in making a cast; cellucotton or wood flour is more suitable for making molds. Clays are satisfactory for incorporating in latex mixes where the cast is to be poured into plaster molds. White clays and zinc oxide counteract the darkening of set latex when exposed to sunlight.

Unvulcanized or common latex must be vulcanized by heat in one form or another to preserve the rubber. It is done after the rubber has set within the mold and generally before the cast is removed. A rubber mold can be vulcanized on the pattern unless the pattern is made of wax. Unvulcanized latex has a tendency to stick to the pattern or mold unless thoroughly dry and vulcanized; therefore, unvulcanized rubber casts are vulcanized while still in the mold.

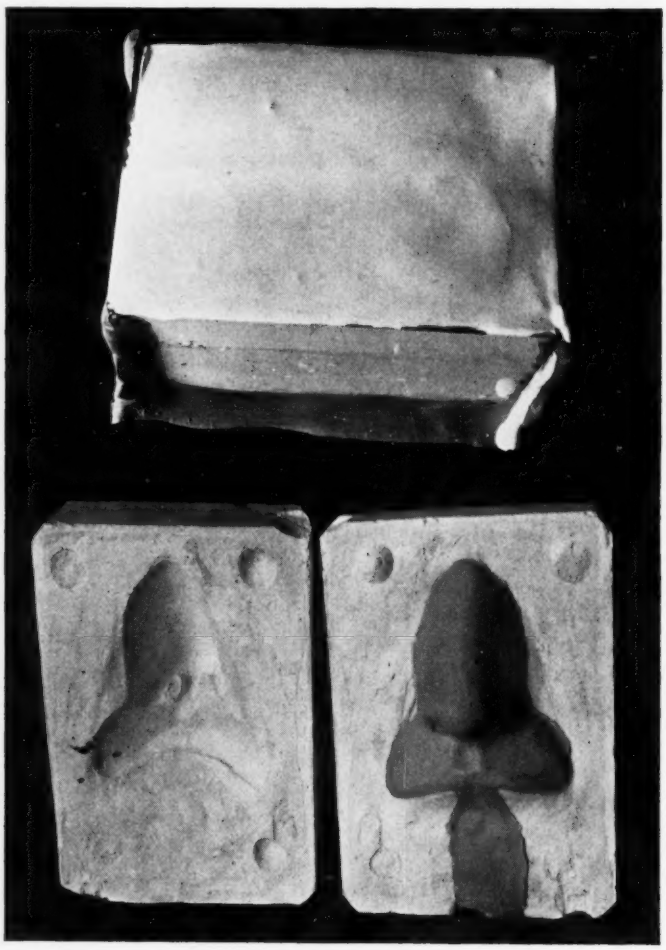

Fig. 4.—*A*. The filled glass box constituting the completed two-piece mold.
B. The mold is opened and the wax cast is removed. If there are undercuts in the cast it may be necessary to destroy the cast to open the mold. In such a case boiling water is poured over the closed mold. The mold will open but the wax cast will be melted.

Vulcanized Latex.—The moulage worker and prosthetist can save considerable time and effort by using vulcanized latex. This is identical in appearance with unvulcanized or common latex; however, it acts in an entirely different manner toward the mold. It will not stick or adhere to plaster and similar patterns or molds when it has set or coagulated. Nevertheless, it should not be removed from the pattern or mold until comparatively dry; otherwise the cast will not retain its shape.

Fillers.—Cellucotton, wood flour, clays, and similar fillers may be added to vulcanized latex in making rubber molds. Since the latex is vulcanized as a liquid before shipment, additional vulcanization is unnecessary. Clays may be added to the prosthetic cast to prevent darkening in sunlight. It should be remembered, however, that the more clay that is added the more translucency is lost. For prosthetic work Bulbulian recommends the addition of 3 Gm. of zinc oxide in a water dispersion to 200 c.c. of vulcanized latex (Vultex H 235). According to tests this will not prevent darkening of the latex. Furthermore, this additional zinc oxide may produce cracking of the resulting prosthesis, since Vultex H 235 already contains some zinc oxide. The cracking, however, may not occur for some months. A white clay filler would be more suitable in this case. The clays do not have the opacity or covering power of zinc oxide; therefore, more than 3 Gm. of clay to 200 c.c. of rubber may be used. This is 1½ per cent. White China clay and Dixie clay suggest themselves for this purpose. Dixie clay, when used in great quantities, not only produces opacity but causes a stiffening of the resulting prosthesis. Titanium dioxide is considered the whitest of the common fillers and possesses a high degree of fastness to light and vulcanization. It is a bright white powder free of yellow tinge; it does not affect the aging of the rubber, but is rather opaque and has considerable covering power. Therefore it must be used in small quantities and should be ground thoroughly in water before use.

The General Latex and Chemical Company* manufactures an excellent clay filler already dispersed in water that is most suitable for prosthetic work. It is their No. H 222, Part B, and can be used in conjunction with Vultex F 293 or H 235. It may be compounded in the Vultex up to 10 per cent without obtaining great opacity or loss of translucency. Of course, this depends on the color and opacity to be produced.

An excess of fillers and sulfur causes rubber to become stiff on drying. A formula for prosthetic work should not include enough of these ingredients to be objectionable. In some cases, however, extreme lightness is desirable. The stiffening of the prosthesis can be offset by pouring a thin rubber coating into the mold, and by then forcing sponge rubber or foamed latex between the thin layers. This produces a lifelike prosthesis that lends itself to trimming of seam lines and yet remains sufficiently flexible to move on the flesh as different expressions are assumed. Sponge rubber alone does not lend itself to trimming and is not as natural in appearance. It does allow the penetration of air through its pores and this has some advantages. Sponge or foamed rubber will be discussed more fully later in this text.

A simple formula containing vulcanized latex is as follows:

Vultex F 293	10 parts by volume
Filler H 222, Part B	1 part by volume
Pontamine fast red 8 B1 (in water)	q.s.

Rubber latex turns slightly yellow on setting and drying. Caucasian skin color generally is obtained by mixing reds and yellows. Since the latex itself is light yellow on drying, only the red is added. Other reds than the Pontamine

*Cambridge, Mass.

fast red mentioned can be used. Colors and coloring of the finished prosthesis will be discussed in detail in a future article. It should be mentioned, however, that the latex stock solution is given a light basic color in the latex mix to resemble a flesh color. This is done with water-soluble dyes having an alkaline reaction and containing no copper. The final coloring is done with oil and alcohol-soluble dyes, not with pigments as is the common belief. Oil-soluble dyes penetrate the dry rubber and blend in a soft homogeneous manner. The dye-colored prosthesis can be washed and scrubbed with a stiff brush immediately after coloring without any loss of color. If pigments were used they would be scrubbed off because they only lie on the surface. Alcohol-soluble dyes do not penetrate the rubber nor do they blend or bleed out into the rubber. When the prosthesis is colored properly, the necessity for the final use of cosmetics becomes lessened or eliminated.

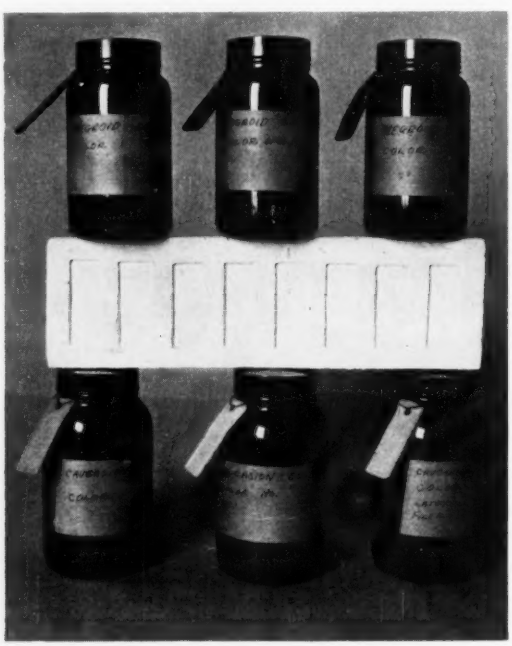

Fig. 5.—Rubber stock solutions of both Caucasian and Negroid skin colors. The plaster block between the bottles is used to obtain dried samples of the various colors. The depressions in this block are numbered from one to eight for specifying the intensity of color.

Pliability.—As already mentioned, the finished prosthesis should possess enough pliability when used as an artificial part on the face to move with the remaining features as they assume various expressions. A thick, solid prosthesis would not have sufficient pliability to move with the features without separating from the skin at corners where the prosthesis joins the face. An example of this inflexibility would be noted in the outer borders of the upper lip. Three alternative remedies for this defect may be considered. The prosthesis can be made rather thin and thus lend itself more readily to shrinkage and distortions, or a thin shell may be filled with sponge rubber to give body to the resulting prosthesis. In the latter instance pliability still remains, in spite of the fact that

the prosthesis seems thick. The third alternative is the use of a softening medium in the latex mix, such as castor oil, mineral oil, and stearic acid. These oils must be converted into emulsions or suspensions in aqueous mediums before being incorporated into the latex mix. Soft soap may be used for this purpose as could any of the substances of a colloidal nature which serve as stabilizers of rubber latex itself, such as casein. Complete information on this subject will be found in most books on rubber latex.

Sponge Rubber.—Prostheses have been made of sponge rubber alone and are rather light and pliable. Air will pass through them, and for this reason it is assumed that they would be more comfortable to wear, since they give the tissues a chance to breathe beneath the artificial organ. In the author's opinion the prosthesis made of sponge rubber is impractical in most cases. It does not lend itself to the trimming of seam lines after removal from the mold. It becomes distorted and remains so. If cosmetics and adhesives are applied to the surface the air cannot pass through the pores. Sponge rubber does not have a natural appearance. Sometimes a comparatively stiff rubber prosthesis is desired, such as a hand, for example. As a matter of fact, a microcrystalline wax is poured into the prostheses of hands to increase the stiffness susufficiently to allow the wearer to hold a pen or pencil for writing or tableware for eating. If the rubber fingers are filled with this plastic wax, they can be moved to adequate positions for holding such implements. The wax core, which in itself is pliable but remains where placed without cracking, is thick enough for this purpose. Microcrystalline waxes will be discussed in detail in another article.

When pliability and lightness are desired in prostheses for parts of the face, both solid and sponge rubbers are used. The technique for producing such a prosthesis is simple: The formula having the correct basic flesh color (monochrome) and other compounding ingredients is poured into the plaster mold and allowed to remain until a deposit or coating of sufficient thickness is formed. This depends on the dryness of the mold, the size of the resulting prosthesis, and the time the mixture remains in the mold. The longer it stays in the mold the thicker the deposit. In small prostheses a coat of sufficient thickness will be built up in a dry mold within three or four minutes or less. The rubber is then poured from the mold and the mold allowed to drain. This can be done by placing the mold on the spout of the bottle from which the rubber was poured. As the mold drains, a small amount of the same formula is put in a dish and agitated by an egg beater until it is well foamed. The rubber should gain at least three times its volume after beating. It will be sluggish and will not pour as readily into the mold. A small amount may be poured into the opening of the mold, however. A large rubber bulb is then used to force this sponge rubber into the mold and the process is repeated until the mold cannot hold more rubber. The mold is then placed in a warm place until the cast or prosthesis is thoroughly dry. This should take place in from twelve to twenty-four hours when the mold is placed in a drying cabinet or on a heated radiator. If the cast is removed too soon it will shrink and become distorted.

Prostheses that are to be enlarged by soaking in benzine[8] for further casting to offset shrinkage should not be cast in sponge rubber, because they disintegrate more quickly in the expanding agent than solid rubber does.

Preparing Sponge Rubber.—Sponge or foamed rubber can be prepared from ordinary latex containing the compounding and vulcanizing ingredients, or from vulcanized latex. An emulsifying agent such as liquid soap is added to

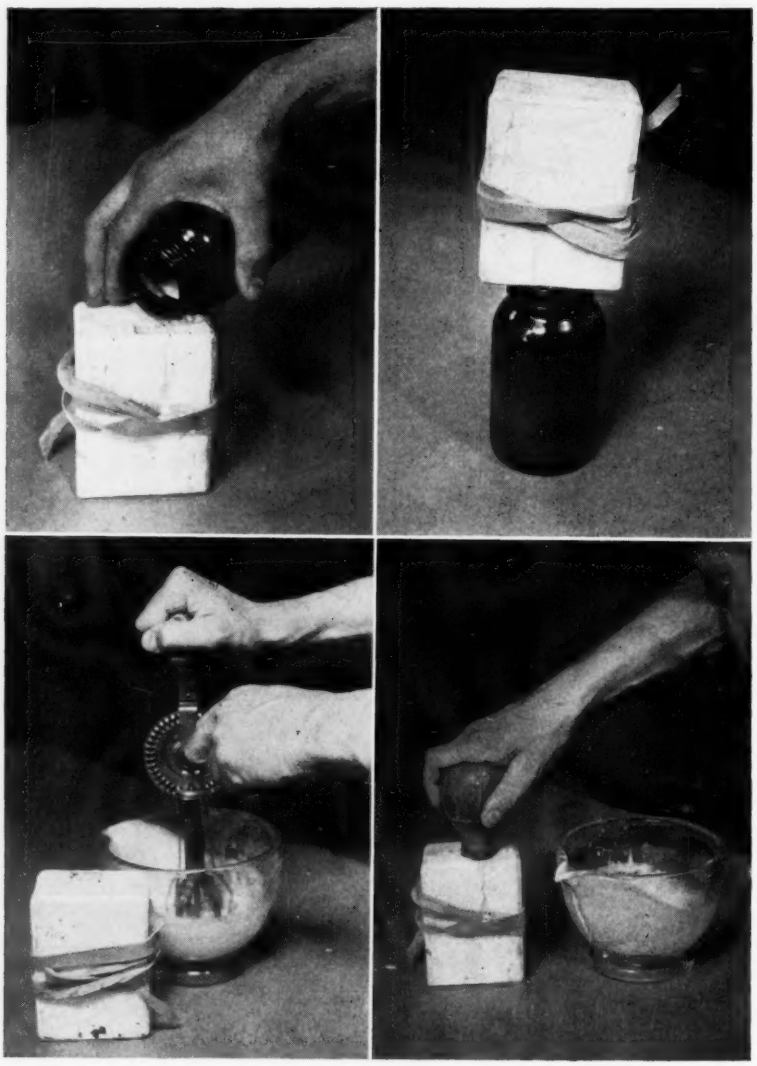

Fig. 6.—*A.* In making a prosthetic rubber nose the cast is poured from the desired stock solution.

B. If it is desirable to use a sponge rubber center for the prosthesis, the rubber is poured from the mold back into the bottle after being in the mold for a minute or more.

C. Part of the rubber from the same stock solution is foamed with an egg beater to form the sponge rubber.

D. Some of this spunge rubber is poured into the mold and forced down with a rubber bulb. From time to time the process must be repeated.

the latex formula in order to stabilize the foam; a setting agent, which is a coagulant having a delayed action, is also added to set the foam. The ingredients are adjusted in the formula, and treatment of the mold is carried out in a manner so that the foam produced by beating, whipping, or blowing the latex mixture is stabilized in this condition. Sodium bicarbonate also may be employed for foaming rubber by effervescence, but this method is not as dependable as

the introduction of air into the latex mix by mechanical means. The soap and salt are added to the mix so that the bubbles will not burst or subside until the setting agent coagulates the whole. The same metallic salts may be used in sponge rubber as in the mixes for coagulating coats or deposits on nonporous molds, such as those of agar, metal, or rubber. After the bubbles and film have set, the rubber is dried. By adjusting the proportion of the setting agent and the temperature of the mold, the time in which the latex froth will set can be controlled readily. Some of the common salts used in sponging latex are calcium sulfate, magnesium sulfate, and calcium chloride. The proportion used is necessarily small, approximately 1/40 part to the latex mix. The salts should be dissolved in water before being added to the latex mix.

SOFTENERS AND PLASTICIZERS

Sometimes it is desirable to have a prosthesis that is solid as well as soft, but made without sponge or foamed rubber. For this purpose softeners or plasticizers are incorporated into the latex mix before it is poured into the mold. These softening agents render the rubber impression more pliable, a desirable feature in facial prostheses which must move with the muscles.

The following are two formulas suitable for this purpose:

No. 1

STEARIC ACID OR PARAFFIN FORMULA

Stearic acid or paraffin	4 Parts
Oleic acid	1 Part
Water	5 Parts
Caustic potash	3 Per cent of weight of stearic acid or paraffin
Ammonia solution	3 Per cent of 26° Be

The stearic acid or paraffin is melted with the oleic acid while stirring vigorously. These ingredients are stirred into the water containing the caustic potash. The ammonia is added while the stirring is continued. The emulsion is carried out best at 70° to 80° C.

No. 2

MINERAL OR PARAFFIN OIL FORMULA

A mineral or paraffin oil emulsion may be prepared by adding 2 to 5 per cent of oleic acid to the oil, and then by rapidly stirring the mixture into water containing 1 per cent of 26 Be ammonia solution. This emulsion may be prepared cold with a concentration of 50 to 60 per cent of oil.

Up to 10 per cent of the paraffin or stearic acid emulsion may be used in the latex mix; but if 2 per cent is exceeded, blooming usually occurs. The mineral oil emulsion does not bloom as readily as the paraffin formula and as much as 10 per cent may be used; however, a staining is more likely to occur with this formula than with paraffin.

For prosthetic work it is advisable to mix stock solutions of one or both of these plasticizers to be incorporated at the time a latex mix is prepared for a specific prosthesis.

Coagulum Deposit.—As previously mentioned, it may be necessary in producing the cast to build up a coagulum deposit on nonporous agar and similar molds to develop sufficient thickness for the cast to be of practical value. The

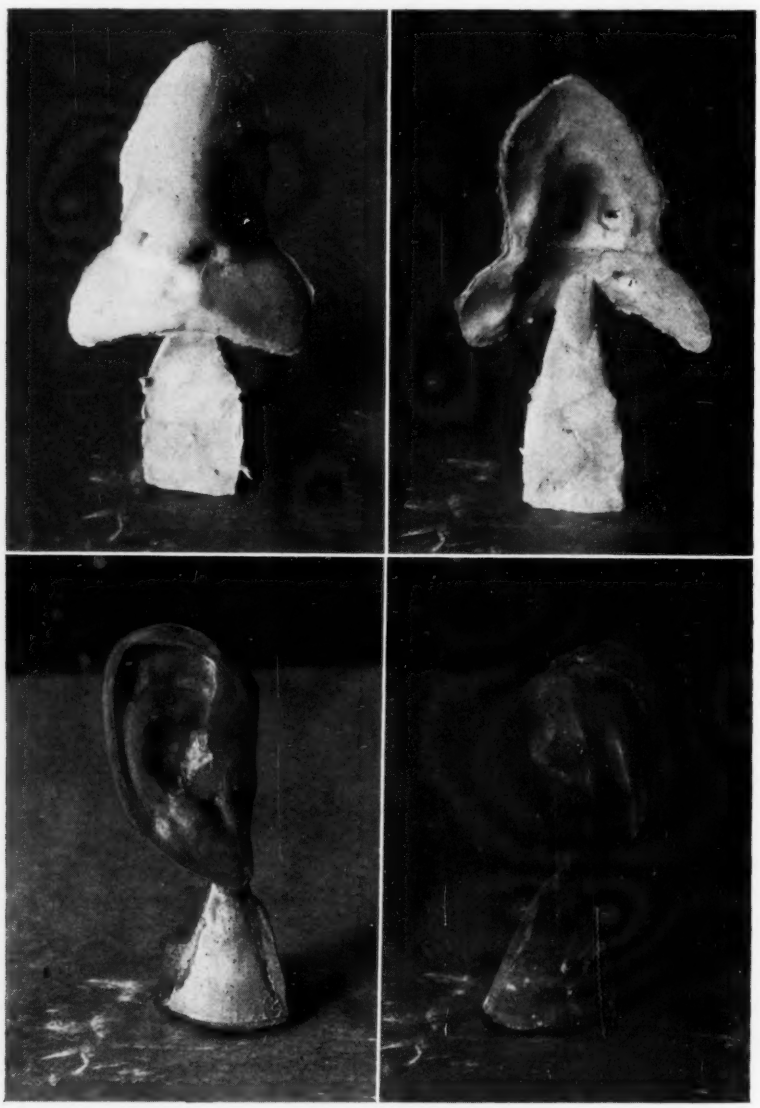

Fig. 7.—*A.* When the cast is completely dry, the mold is opened and the rubber prosthesis is removed. This illustration shows the front view of the untrimmed prosthesis immediately after removal from the mold.
B. The back view of the same prosthesis showing the area to be joined to the skin.
C. A prosthetic ear for a Negro immediately after removal from the mold.
D. Some of this sponge rubber is poured into the mold and forced down with a rubber trimmed with hot instruments, and smoothed off with benzine, and tinted before being attached to the patient.

coagulating agent can be incorporated into the agar-molding composition, or it may be applied to the surface of the mold. A coagulating agent for mixing with agar compositions is magnesium sulfate (epsom salts), because it has no detrimental effect on the agar. Plaster of Paris or unglazed terra cotta molds need no coagulating agents, since these porous molds absorb the water from the latex

mix and thereby build up a coagulum deposit next to the mold surfaces. Metal molds can be used for rubber casting, since a coagulum deposit can be built up on a metal mold by the application of heat to the mold before the deposit is made. A rubber mold can be used to cast rubber, because a coagulum deposit can be built up on a rubber mold which has been treated previously with a coagulant and a separating medium. A wax mold can be treated with a coagulating agent only, as a separating medium is unnecessary.

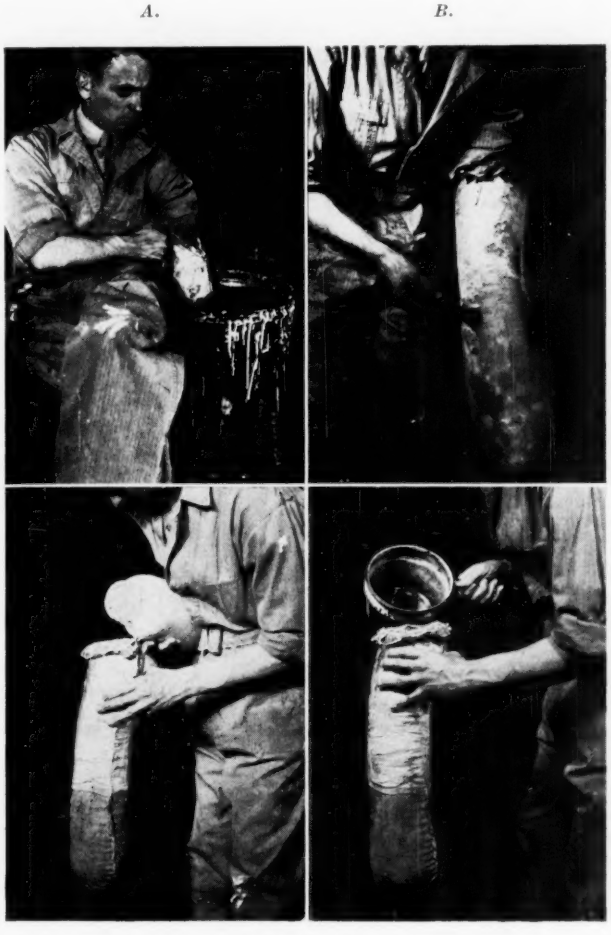

Fig. 8.—The making of a prosthetic rubber hand and arm. *A*. The agar composition is poured into a cloth bag. After it has cooled to near its setting point, the hand and arm are inserted into the bag. The bag is placed in ice water where it remains until the agar has completely set. *B*. Before removing the hand, a slit is made opposite the wrist in the agar mold and cloth bag. The hand is then removed. *C*. The rubber latex composition is poured into the bag where it is allowed to stand until a coagulum deposit of about one eighth of an inch is built up next to the mold. The excess rubber is then poured out. *D*. After the coagulum deposit has fully set, but not dried, a melted microcrystalline flexible wax is poured as a core into the rubber cast while the cast is still in the mold.

Where a thick deposit is necessary it is advisable to use a concentrated latex. The thickness of the deposit is dependent on a number of factors, some of which can be controlled at the compounding stage. If unvulcanized latex is used, the simple addition of the vulcanizing ingredients increases the viscosity of the latex. The addition of 2 or 3 per cent of zinc oxide in particular has been found to have an appreciable thickening effect on latex. If more than 5 per cent is added,

a complete coagulation of the entire mixture may result. Some soluble metallic salts, such as calcium sulfate in small quantities (0.3 per cent of the dry rubber content), cause a slight thickening and render the latex sensitive to local heating. By this addition or pretreating of the latex mix, a firm thick coagulum may be built up when the mixture comes in contact with hot surfaces. Salts of magnesium and zinc also serve for this purpose.

Where a thick coagulum deposit is necessary on nonporous molds, the mold may be dipped, sprayed, or painted with dilute acetic acid. Often this is done after the first thin layer of latex has been deposited. The second layer will then be rather thick. After the deposit has completely coagulated, the excess acid should be washed away with water.

The coagulants, commonly employed are acetic acid, formic acid, calcium chloride, acetate, nitrate or formate, zinc chloride, and ammonium acetate in water. Acetone or alcohol solutions are also used, depending upon the molding material and the conditions under which it is desired to effect a coagulation. Methyl alcohol has been of value, because of its readily wetting properties. Two or more coagulants have been used together, as, for example, calcium chloride (2½ per cent) in equal parts of methyl alcohol and water. In fact, I have found calcium chloride and acetic acid among the best coagulants for prosthetic work, according to the problem to be solved. The salt was used in the mix and the acid was applied to the surface of molds. The coagulating agent may be painted or sprayed on the surface of the mold, or it may be poured into closed molds and drained out. It may or may not be dried on the form. A coagulant should be chosen and applied in such a manner, however, that the rubber will be coagulated immediately, so that there will be little tendency for the latex to flow or cause "sags." "Sags" may be the result of two thin coagulated surfaces of latex between which is a deposit of uncoagulated latex. They may also be produced by coagulating one layer over another in the same mold, a practice which is not always good. It is best to try to build up a deposit of sufficient thickness in one pouring. After the deposit is made it must be dried. In the case of prosthetic rubber hands, a melted microcrystalline wax is poured into the hollow hand before it is removed from the mold to prevent shrinkage of the rubber and to make the hand of practical use. In such a case an unvulcanized latex formula may be used, as the hot wax will vulcanize the thin rubber deposit.

STEPS IN MAKING A PROSTHETIC HAND

A practical example of building up a coagulum deposit is obvious when a rubber pattern is to be made from an agar mold for a prosthetic hand and arm. Briefly, the procedure is as follows. A one-piece agar mold is made of the donor's hand and arm by filling a cloth bag with melted agar composition containing epsom salts. The composition should be cooled to a point where it is comfortable to the donor. His hand and arm are inserted into the bag and the bag is placed in a deep bucket of ice water until the agar sets. If started at the right temperature this should take from one-half to three-quarters of an hour. When the agar has set completely the hand is withdrawn. Before withdrawing the hand, however, a slit should be cut on one side of the bag of agar composition opposite the wrist to allow the mold to spread, so that the largest part of the hand

can pass through the small opening formerly occupied by the wrist. After the hand is removed a gauze bandage is tied around the agar mold at the wrist to close the expansion slit. A coagulating agent is poured into the bag to further treat the surface of the mold. This is drained out and hot air forced in to dry the surface. The mold is then filled with rubber latex which is allowed to stand until a deposit of one-eighth inch is built up on the sides of the agar mold. This deposit is caused by the epsom salts in the agar, plus the surface coagulant. After a one-eighth inch deposit is built up the remaining rubber is poured out

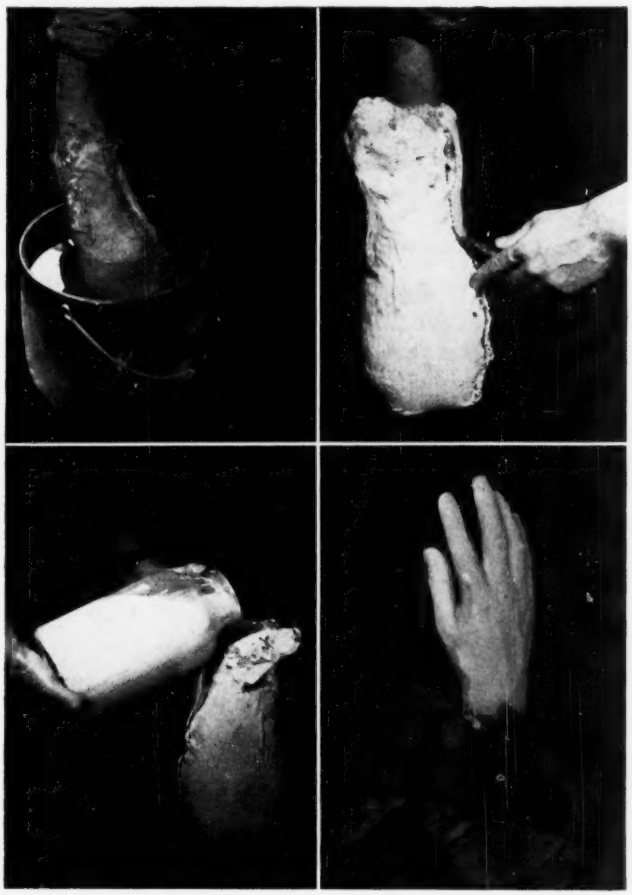

Fig. 9.—The making of a prosthetic rubber hand. *A*. The agar mold is placed in ice water. *B*. The mold is cut opposite the wrist. *C*. The rubber is poured into the mold. After setting, the wax is poured into the cast to form the core. *D*. The cloth bag is removed, and then the agar mold is cut into blocks and broken away from the cast. The rubber hand is still wet and must dry on the wax core. The seam line caused by cutting the agar mold to release the hand is plainly visible on the wrist of the rubber cast. This is trimmed off with hot instruments after the cast has fully dried.

and the mold is placed on its open end over a collecting pan to permit it to drain. When the rubber stops dripping, a hair drier, which is a fan blowing heated air through a small opening, is placed in the open end of the cast to aid in further coagulation of the cast while it is still in the mold. After the rubber cast has coagulated sufficiently to stand handling of the edges, it is filled with a microcrystalline wax to serve as a core to prevent shrinkage of the cast when the agar mold is removed. The rubber at this stage should not be com-

pletely dry, or cracking will result. If the wax is not poured at this time the cast will continue to dry from the inside and considerable shrinkage will take place. The agar mold is removed after the wax has set completely but before the rubber is dry. On removal, it will be found that seam lines were formed on the rubber where the mold was cut opposite the wrist. These are dressed off with heated instruments and all other defects are repaired. Although such a hand can be used as a prosthesis for the patient, it is far better to use it as a pattern for making a one-piece plaster mold so that many duplicates can be made. This is done by applying plaster to the rubber hand to form the mold. After the mold is made, it is placed in an oven and heated until the wax core of the cast is melted. The wax is then poured out. A thin rubber shell of a hand incased in a plaster mold remains. By grasping the rubber edges the collapsible hand may be pulled from the mold. Duplicate hands are then made in the same mold by pouring rubber into it, allowing the deposit to be built up, and pouring out the excess latex. The rubber cast may be dried from within by using the hair drier. The duplicate hand is removed and the process repeated until a sufficient number of prostheses are obtained. The flexible microcrystalline wax is poured into the rubber hands after removal from the plaster mold to give them stiffness and to make them practical for grasping objects. The fingers of the prosthesis are simply bent with the natural hand into the correct position for practical use. The object to be used is then placed into the prosthetic hand with the natural hand.

The prosthetic hand is colored with oil-soluble dyes for softly blended effects and with alcohol-soluble dyes for detail. The oil-soluble dyes bleed into the rubber and permeate the color through the entire thickness of rubber. Alcohol-soluble dyes do not bleed into the rubber but remain where they are placed; therefore, they can easily be washed from the surface. This can be prevented by the application of a fine coat of semimatte lacquer or by the application of a latent rubber solvent to the prosthesis. Such a solvent opens the pores of the rubber temporarily to absorb the dye. The solvent evaporates quickly without causing the surface of the prosthesis to lose detail. Mixtures of turpentine and benzine serve well for this purpose. The turpentine acts slowly on the rubber, whereas the benzine acts rather quickly; therefore, the turpentine is used as a retarding agent for the benzine. Immediately after these materials are applied, the prosthesis can be washed with soap and water to see if the desired effect thus obtained will be permanent.

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